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Ph.D. Thesis

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Abbreviations

20-HE	20-Hydroxyecdysone
ACR	Acute-to-chronic ratio
ADBI	Celestolide
AHTN	Tonalide
AGH	Androgenic gland hormone
BDE	Brominated diphenyl ether
BPA	Bisphenol A
CHH	Crustacean hypoglycemic hormone
DCP	3,5-Dichlorophenol
DCA	3,4 Dichloroaniline
p,p'-DDD	1,1-Dichloro-2,2-bis(4-chlorophenyl)ethane
p,p'-DDE	1,1-Dichloro-2,2-bis(4-chlorophenyl)ethylene
p,p'-DDT	1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane
DEHP	Di(2-ethylhexyl)phthalate
DEP	Diethyl phthalate
DES	Diethylstilbestrol
E1	Estrone
E2	17β-Estradiol
EC_x	Effective concentration for x % of the test organisms
EDC	Endocrine Disrupting Chemical
EE2	17α-Ethinylestradiol
ELISA	Enzyme-linked immunosorbent assay
GIH	Gonad-inhibiting hormone
GSH	Gonad-stimulating hormone
HHCB	Galaxolide
ICI	Imperial Chemical Industries
ISO	International Organisation for Standardisation
JH-III	Juvenile hormone-III
LC	Lethal concentration for x % of the test organisms
LDR	Larval development ratio
LOEC	Lowest observed effect concentration
MF	Methyl farnesoate
MIH	Molt-inhibiting hormone
MK	Musk ketone
MOIH	Mandibular organ-inhibiting hormone
NOEC	No observed effect concentration
NP	Nonylphenol
NPEO	Nonylphenol ethoxylate
OCDF	Octachlorodibenzofuran
OECD	Organisation of Economic Co-operation and Development
OP	Octylphenol
PAH	Polycyclic aromatic hydrocarbons
PBDE	Polybrominated diphenyl ether

PCB	Polychlorinated biphenyl
PCDF	Polychlorinated dibenzofuran
Ph.D.	Philpsophiae doctor
PoA	Ponasterone A
RH	Rohm and Haas Co.
STP	Sewage treatment plant
TBP	2,4,6-Tribromophenol
TBBPA	Tetrabromobisphenol A
TBT	Tributyltin
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
VIH	Vitellogenesis-inhibiting hormone
VSOH	Vitellogenesis-stimulating ovarian hormone

Abstract

New and updated test methods to detect and characterise endocrine disrupting chemicals are urgently needed for the purpose of environmental risk assessment. Although endocrine disruption in invertebrates has not been studied as extensive as in vertebrates, in particular in fish, numerous reports on effects of potential endocrine disrupters on crustacean development and reproduction have been published since the beginning of the nineties. However, most crustacean toxicity test protocols routinely used so far have not been designed with endocrine-specific endpoints in mind. The main objectives of the present Ph.D. project were:

1) to develop a fully synthetic saltwater medium suitable for laboratory culturing of marine copepods including their feeding organism as well as for toxicity testing
2) to identify sensitive endpoints related to growth, development and reproduction of the pelagic calanoid copepod *Acartia tonsa*
3) to elucidate the applicability of those endpoints for testing sublethal effects of model compounds
4) to investigate these model compounds in a second species, the benthic harpacticoid copepod *Nitocra spinipes*
5) to study the model compounds *in vitro* for ecdysteroid agonistic/antagonistic activity with the ecdysteroid-responsive *Drosophila melanogaster* B_{II} cell line
6) to draft an OECD guideline proposal for testing of chemicals based on the experimental work performed within this study

In preliminary investigations with *A. tonsa* were studied various parameters related to processes regulated by hormones such as growth, molting, sexual maturation and reproduction. The primary endpoints were larval development ratio, egg production and sex ratio. Exposure experiments were conducted with naturally occurring and synthetic vertebrate and invertebrate hormones as well as compounds known to act as endocrine disrupters in vertebrates. Larval development ratio was identified to be a remarkably sensitive endpoint. The larval development test with *A. tonsa* is rapid, cost-effective, easily to perform and results in full concentration-response relationships allowing the determination of effective concentrations (ECx).

After having demonstrated that larval development of *A. tonsa* was a very sensitive endpoint for evaluating effects of chemicals that might interfere with the endocrine system of crustaceans, the larval development test has been applied to two groups of emerging environmental contaminants, brominated flame retardants (BFRs) and

synthetic musks, known or suspected to be potential endocrine disrupters. In parallel, these compounds were investigated in a full life-cycle test with *N. spinipes*. This test allows studying larval development ratio as well as other individual life-table endpoints, which enable the calculation of population-level endpoints, e.g. the intrinsic rate of natural increase. In *N. spinipes*, larval development ratio has been proven to be the most sensitive endpoint too. The knowledge about the impact of BFRs on aquatic organisms is still limited. A primary contribution of the present work with BFRs was to establish data on their (sub)chronic toxicity towards marine copepods.

To discriminate between general toxicological and endocrine-mediated toxic effects, the model compounds were assessed *in vitro* for ecdysteroid agonistic/antagonistic activity using the *Drosophila melanogaster* B_{II} cell line assay. The pentabrominated diphenyl ethers BDE-99 and BDE-100 showed weak ecdysteroid antagonistic activity. Thus, they are able to interfere with a crucial physiological mechanism in arthropods, a property that only is known for a very few substances. The combination of *in vitro* assays and (sub)chronic copepod tests, as applied in this study, is a valuable tool when screening chemicals suspected to be specifically toxic, in particular, to interfere with the endocrine system.

The results of the experimental work as well as the literature survey demonstrated clearly that marine copepods such as *Acartia tonsa* and *Nitocra spinipes* are suitable and very sensitive test organisms to study sublethal effects of specific toxic chemicals including compounds suspected to be potential EDCs.

Another outcome of this study, which is of particular importance for environmental risk assessment of chemicals, is the OECD Draft Guideline for Testing of Chemicals - Proposal for a New Guideline, Calanoid Copepod Development and Reproduction Test with *Acartia tonsa.*

Resumé (in Danish)

Ved risikovurdering af kemikalier er der et stort behov for nye og opdaterede testmetoder til påvisning og karakterisering af hormonforstyrrende stoffer. Selvom hormonforstyrrelser i invertebrater er mindre velundersøgte end i vertebrater, specielt i fisk, findes der en lang række studier af forskellige effekter af potentielt hormonforstyrrende stoffer på udvikling og reproduktion hos krebsdyr. De fleste rutinemæssigt anvendte testmetoder med krebsdyr er dog ikke blevet designet specifikt med henblik på hormonsystem-relaterede endpoints. Formålet med dette Ph.D. projekt har været:

1) at udvikle et fuldt syntetisk marint medie til laboratoriedyrkning af marine copepoder og deres fødeorganismer samt til toksicitetstests
2) at identificere sensitive parametre relateret til vækst, udvikling og reproduktion hos den pelagiske calanoide copepod *Acartia tonsa*
3) at vurdere anvendeligheden af disse parameter til detektion af subletale effekter af modelstoffer
4) at undersøge effekter af de samme modelstoffer på en anden copepod, den bentiske harpacticoid *Nitocra spinipes*
5) at teste modelstofferne *in vitro* for deres ecdysteroid agonistisk/antagonistisk aktivitet ved brug af den ecdysteroid-responsive *Drosophila melanogaster* B_{II} cellelinie
6) at udarbejde et udkast til en standard-testmetode for undersøgelse af kemikalier, baseret på det eksperimentelle arbejde udført i dette projekt

I indledende forsøg med *A. tonsa* blev undersøgt forskellige parameter, som er relateret til processer, der er under hormonal kontrol. Sådanne processer er blandt andet vækst, hudskifte, kønsmodning og reproduktion. De vigtigste endpoints var larveudviklingsratio, ægproduktion og kønsratio. Eksponeringsforsøg blev udført med både naturligt forekommende og syntetiske vertebrat- og invertebrathormoner, såvel som stoffer kendt eller mistænkt for at forstyrre hormonsystemer i vertebrater. Larveudviklingsratio blev identificeret som et specielt følsomt endpoint. Larveudviklingstesten med *A. tonsa* er hurtig, billig og let at udføre og resulterer i fulde koncentrations-respons-sammenhænge, hvilket giver mulighed for beregning af effekt-koncentrationer (ECx).

Efter påvisningen af larveudviklingen af *A. tonsa* som et meget følsomt endpoint for tests med kemikalier, som muligvis påvirker det endokrine system af krebsdyr, blev

larveudviklingstesten anvendt på to grupper af nye miljøfremmede stoffer, bromerede flammehæmmere (BFRs) og syntetiske moskusstoffer. Begge stofgrupper er kendt for deres hormonforstyrrende egenskaber i vertebrater. Samtidigt blev disse stoffer undersøgt i en livscyklustest med *N. spinipes*. I denne test blev larveudviklingen såvel som andre individuelle livs-forløb endpoints undersøgt, hvilket muliggjorde beregningen af et endpoint på populationsniveauet, den potentielle populations-vækstrate. Ligesom hos *A. tonsa* var larveudviklingsratioen den mest følsomme parameter hos *N. spinipes*. Viden om skadelige effekter af BFRs på akvatiske organismer er stadig begrænset. Etableringen (sub)kroniske toksicitetsdata for BFRs med marine krebsdyr er derfor et væsentlig nyt vidensbidrag.

For at kunne skelne mellem endokrine og andre typer effekter blev modelstofferne undersøgt *in vitro* for deres ecdysteroid agonistisk/antagonistisk aktivitet ved brug af *Drosophila melanogaster* B_{II} cell linie-testen. De polybromerede diphenyl-ethere BDE-99 and BDE-100 udviste en svag ecdysteroid antagonistisk aktivitet. De er derved i stand til at vekselvirke med en afgørende fysiologisk mekanisme i arthropoder - en egenskab, som kun er kendt for meget få stoffer. Kombinationen af *in vitro*-metoder og (sub)kroniske copepodtests, som anvendt i dette studie, er et værdifuldt værktøj til screening af kemikalier, der er mistænkt for at virke specifikt toksisk herunder med direkte virkning på det endokrine system.

De eksperimentelle resultater såvel som litteraturstudiet viser klart, at marine copepoder som *Acartia tonsa* og *Nitocra spinipes* er velegnede og følsomme testorganismer til undersøgelser af subletale effekter af specifikt virkende stoffer inklusive potentielt hormonforstyrrende stoffer.

Et andet væsentligt resultat, som er af betydning for risikovurderingen af kemikalier, er forslaget til en ny OECD testmetode: Draft Guideline for Testing of Chemicals - Proposal for a New Guideline, Calanoid Copepod Development and Reproduction Test with *Acartia tonsa*.

Preface

The present dissertation was prepared at Environment & Resources, Technical University of Denmark, as part of the fulfilment of the Ph.D. degree requirements. The experimental work was carried out from February 1999 to October 2002 with Associate Professor K. Ole Kusk, Environment & Resources, Technical University of Denmark, as supervisor and Professor Bent Halling-Sørensen, Department of Analytical Chemistry, The Danish University of Pharmaceutical Sciences, as co-supervisor. A part of the experimental work was conducted during a five-month research stay at the Laboratory for Aquatic Environmental Chemistry, The Institute of Applied Environmental Research, Stockholm University. The project was funded by the Technical University of Denmark.

The thesis comprises a synopsis focussing on sublethal effects of (potential) endocrine disrupting chemicals in crustaceans with special emphasis on copepods and molting, seven papers published in peer-reviewed journals as well as a draft OECD guideline for testing of chemicals using *Acartia tonsa*, which is primarily based on experiments conducted within this study and a follow-up research project.

The titles of the papers and the OECD draft guideline are:

[1] **Wollenberger, L.**, Halling-Sørensen, B., and Kusk, K.O. 2000. Acute and chronic toxicity of veterinary antibiotics to *Daphnia magna*. *Chemosphere* 40: 723-730.

[2] Kusk, K.O. and **Wollenberger, L.** 1999. Fully defined saltwater medium for cultivation of and toxicity testing with marine copepod *Acartia tonsa*. *Environmental Toxicology and Chemistry* 18: 1564-1567.

[3] Andersen, H.R., **Wollenberger, L.**, Halling-Sørensen, B. and Kusk, K.O. 2001. Development of copepod nauplii to copepodites – A parameter for chronic toxicity including endocrine disruption. *Environmental Toxicology and Chemistry* 20: 2821-2829.

[4] **Wollenberger, L.**, Breitholtz, M., Kusk, K.O. and Bengtsson, B.-E. 2003. Inhibition of larval development of the marine copepod *Acartia tonsa* by four synthetic musk substances. *The Science of the Total Environment* 305: 53-64.

[5] Breitholtz, M., **Wollenberger, L.** and Dinan, L. 2003. Effects of four synthetic musks on the life cycle of the harpacticoid copepod *Nitocra spinipes*. *Aquatic Toxicology* 63: 103-118.

[6] **Wollenberger, L.**, Dinan, L. and Breitholtz, M. 2005. Brominated Flame Retardants: Activities in a Crustacean Development Test and in an Ecdysteroid Screening Assay. *Environmental Toxicology and Chemistry* 24: 400-407.

[7] Breitholtz, M. and **Wollenberger, L.** 2003. Effects of three PBDEs on development, reproduction and population growth rate of the harpacticoid copepod *Nitocra spinipes*. *Aquatic Toxicology* 64: 85-96.

[8] Kusk K.O. and **Wollenberger, L.** 2004: OECD Draft Guidelines for Testing of Chemicals - Proposal for a New Guideline: Calanoid Copepod Development and Reproduction Test with *Acartia tonsa*. Organisation for Economic Co-operation and Development, Paris, France.

The papers are not included in this www-version but can be obtained from the library at Environment & Resources, Bygningstorvet, Building 115, Technical University of Denmark, DK-2800 Lyngby (library@er.dtu.dk).

(In-text references related to the eight papers are highlighted in bold type.)

This project would not have been possible without the assistance and co-operation of a number of people. Hence, I would like to express my special thanks to them. First of all to my supervisor K. Ole Kusk for many years of close and fruitful co-operation, suggestions, critical comments and helpful discussions. I wish also to thank my co-supervisor Bent Halling-Sørensen for encouraging discussions, which helped me very much at the beginning of my Ph.D. work when I sometimes was in doubt about my results.

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I'm much indebted to all colleagues from the Institute of Applied Environmental Research, Stockholm University, for their hospitality that made me feel at home in Sweden already from the very beginning. Bengt-Erik Bengtsson, who enabled the stay

at ITM, should be especially mentioned. Of course, I gratefully acknowledge the close and efficient co-operation with Magnus Breitholtz, his enthusiasm and ideas in our joint work on crustaceans. Also special thanks to Karin Ek for her skilled technical assistance.

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I thank Grete Hansen and Helle Offenberg for rapid providing of books, reports and printed copies of scientific publications as well as their qualified assistance in searching for literature in databases.

I gratefully acknowledge the skilled assistance of Birte Brejl and Torben Dolin in designing figures and graphs for publications and posters.

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Finally, I would like to thank my colleagues from the Ecotoxicology Laboratory at Environment & Resources for the really pleasant working atmosphere. Birthe Ebert, Camilla Hedberg, Signe Qualmann, and Susanne Kruse should be mentioned in particular.

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1 Background and Aim of the Study

Over the last ten years there has been growing concern about low-level exposure of man and wildlife to xenobiotics capable of modulating or disrupting the endocrine system. There is strong evidence that potentially adverse effects occur in wildlife at the individual and population level caused by disruption of one or more endocrine systems. For example, male fish with detectable levels of vitellogenin, a female-specific protein, were seen in various rivers in United Kingdom and the USA [9-11]. Imposex (development of male sexual characteristics by females) and decreased reproductive success in some marine gastropods were causally linked to the exposure to tributyltin (TBT) [12]. A spill of organochlorine pesticides in Lake Apopka, Florida, USA, was assumed to be responsible for morphological abnormalities and impaired reproduction in the alligator population of the lake [13;14].

The term *endocrine disruption* has only be in use since the early nineties [15], although it is very likely that this type of effect has been occurring in wildlife at least as long as humans have been releasing synthetic chemicals into the environment [16]. The most widely used definition of endocrine disruption was agreed upon at the European workshop on the impact of endocrine disrupters on human health and wildlife, held at Weybridge, UK, in 1996 [17] as follows:

> *An endocrine disrupter is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function*.

This definition implies that an endocrine disrupter can be identified only when using *in vivo* test systems because it explicitly refers to adverse health effects in intact organisms. The term potential endocrine disrupter should hence be applied to compounds for which only *in vitro* endocrine activity was demonstrated. This term was also defined at the Weybridge workshop as follows:

> *A potential endocrine disrupter is a substance that possesses properties that might be expected to lead to endocrine disruption in an intact organism.*

From these broad definitions it becomes evident that there is a large range of possible effects and mechanisms of action. Endocrine disrupters are believed to interfere with the functioning of hormone systems in at least three possible ways [18]:

- By mimicking the action of a natural hormone such as estradiol or testosterone, and thereby setting off similar biological response in the body (e.g. via receptor activation)
- By blocking hormone receptors, thereby preventing the action of natural hormones (receptor antagonist)
- By affecting the synthesis, transport, metabolism and excretion of hormones, thus altering the concentrations of natural hormones

Environmental risk characterisation of chemicals relies on results of toxicity tests with organisms representing several trophic levels. This approach can fail in detecting endocrine disrupting effects, since they often occur even at very low concentrations, much below the acute toxic concentrations of the studied compound. The capability of currently available test methods to detect effects related to endocrine disruption has been questioned [19] and the Scientific Committee on Toxicity, Ecotoxicity and the Environment of the European Commission has recognised the need for developing further tests on aquatic invertebrates to address properly endocrine disruption [20].

While a substantial amount of research and method development has been conducted with respect to effects of EDCs in vertebrates, e.g. fish, relative little work has been carried out on aquatic invertebrates so far.

Some hormones are believed to be unique to certain phyla of invertebrates. Examples are ecdysteroids, which regulate molting in insects and crustaceans and the juvenile hormones, which control metamorphosis in insects and are assumed to have similar function in crustaceans [21]. This together with the evidence that several crustaceans have hormones in common with vertebrates, so-called *vertebrate-type steroid hormones* – although their functional roles are unknown – make them suitable species for laboratory testing of potential endocrine disrupters. Moreover, the following criteria should be taken into account when selecting a certain invertebrate species for developing toxicity tests to study the effects of potential EDCs [22]:

- Primary mode of reproduction
- Relative sensitive to a range of chemicals of concern
- Easily to culture and to handle under laboratory conditions

- Short generation time
- Size of organism
- Knowledge of basic endocrinology
- Availability of standard tests protocols, e.g. for acute toxicity testing

Unfortunately, no crustacean species meets all the listed criteria; in particular, our knowledge on the endocrine system of crustaceans is still very limited. Even though, copepods seem to be very promising candidate test species in this context. This was reflected in the OECD Test Guidelines Program [23] in which highest priority has given to the development of survival and reproduction tests with crustaceans including marine, estuarine and brackish copepod species, e.g. *Acartia tonsa* and *Tisbe battagliai.*

In this Ph.D. study, natural and synthetic hormones as well as a wide range of known or suspected EDCs were investigated for their sublethal effects on marine copepod species, the calanoid copepod *Acartia tonsa* and the harpacticoid copepod *Nitocra spinipes*. The main objectives of the work were:

(1) *Development and evaluation of a fully synthetic saltwater medium suitable for laboratory culturing of marine copepods including their feeding organism as well as for toxicity testing*

A synthetic media is always to prefer to a natural one in order to exclude as far as possible any contamination, which might interfere with investigated endpoints. This is of great importance when testing effects of potential EDCs and often not taken into account.

(2) *Identification of sensitive endpoints related to growth, development and reproduction of the pelagic calanoid copepod* A. tonsa

Ecdysteroids similar to vertebrate steroid hormones play a major role in the control of growth, development and reproduction in crustaceans. Therefore, these processes might be vulnerable to endocrine disrupters. Consequently, test systems were set up allowing studying various endpoints such as larval development ratio, sex ratio and egg production. Exposure experiments were conducted with naturally occurring and synthetic vertebrate and invertebrate hormones as well as compounds known to act as

endocrine disrupters in vertebrates *in vitro* or *in vivo*. Moreover, the study focussed particularly on the identification of endpoints suitable for screening purposes.

(3) *Applicability of the identified endpoint(s) for testing sublethal effects of model compounds*

Two groups of model compounds were chosen, brominated flame retardants (BFR) and synthetic musks. Both groups of substances are characterised by their ability to induce various specific toxic effects including endocrine disruption in vertebrates [24-26]. Furthermore, data on their ecotoxicity are scare. Effects of the model compounds on larval development ratio were studied in a partial life cycle test with *A. tonsa* lasting five days.

(4) *Comparative study with a second species, the benthic harpacticoid copepod* Nitocra spinipes*, using the same model compounds*

The BFRs and synthetic musks were investigated in a full life-cycle test with *N. spinipes* lasting three weeks. One of the endpoints was the larval development ratio, which directly could be compared with the results obtained in the *A. tonsa* tests.

(5) *Supplementary investigation of model compounds in an ecdysteroid sensitive* in vitro *test*

Results of *in vivo* methods can give an indication of a certain toxic mode of action of the investigated compound, which can be verified by the results of *in vitro* methods. Therefore, the model compounds were studied *in vitro* for ecdysteroid agonistic/antagonistic activity with the ecdysteroid-responsive *Drosophila melanogaster* B_{II} cell line, in addition.

(6) *Proposal for a new OECD draft guideline using* A. tonsa

Though the majority of crustacean species rely entirely on sexual reproduction for their recruitment, the most often applied international guideline to test for effects on crustacean reproduction to date is based on parthenogenesis in *Daphnia* [27]. Standardised methods for testing chronic toxicity with sexually reproducing species are urgently needed, particularly for the evaluation of endocrine disrupting compounds.

2 The Endocrine Regulation of Crustacean Development and Reproduction

2.1 Overview of Crustacean Endocrinology

The use of hormones to regulate biological processes is a strategy common to vertebrates and invertebrates. Most of the current knowledge of crustacean endocrinology is obtained from studies with larger decapods such as crabs, lobsters, crayfish, and shrimps, and has been reviewed previously [21;28]. The endocrine mechanisms in crustaceans are, however, not very well understood. Basically, environmental stimuli are integrated by the central nervous system; neurotransmitters and neuromodulaters trigger the release of neuropeptides, which in turn control the synthesis of hormones by the endocrine glands. The use of hormone cascades with negative and positive feedback loops to regulate physiological processes is a successful strategy that has been appreciably conserved during evolution [28]. Such endocrine cascades provide a link between the nervous system and the endocrine system. They are also important for balancing actions of different hormones at multiple sites.

In crustaceans, at least four major classes of hormones - steroids, terpenoids, peptides, and amines - are involved in the regulation of sexual differentiation, development, growth, molting, reproduction, and behaviour by a highly complex endocrine system. Other processes like carbohydrate metabolism, colour changes, pigmentation, limb regeneration, osmoregulation, and diapause, are under hormonal control as well.

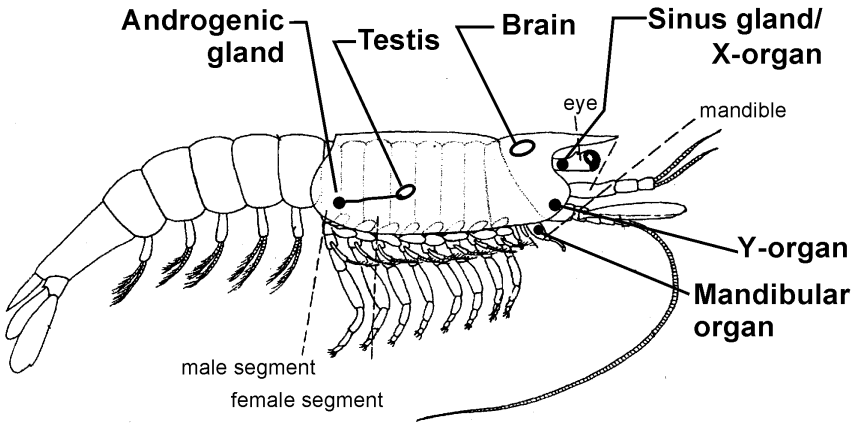


Figure 1 Lateral view of a generalised malacostracan showing the primary endocrine centres of crustaceans (modified from [29])

Table 1 Selected examples of hormones reported to be involved in development and reproduction of crustaceans, chemical types, sources, and functions

Reported hormone	Chemical type	Source	Function/controlled process
Ecdysone	Steroid	Y organs and gonads	Molting, vitellogenesis
Vitellogenesis stimulating ovarian hormone (VSOH)	Steroid (?)	Ovar	Vitellogenesis, female secondary sexual characteristics
Estradiol, testosterone, progesterone	Vertebrate-type steroids	(unclear)	(Functional role under debate)
Methyl farnesoate (MF)	Terpenoid	Mandibular organ	Metamorphosis, reproduction, stimulation of molting
Molt-inhibiting hormone (MIH)	Peptide	X-organ/sinus gland	Ecdysteroid synthesis
Gonad- (vitellogenesis-) inhibiting hormone (GIH or VIH)	Peptide	X-organ/sinus gland	Vitellogenesis
Gonad-stimulating hormone (GSH)	Peptide	Protocerebrum	Female and male reproduction
Mandibular organ-inhibiting hormone (MOIH)	Peptide	X-organ/sinus gland	Molting, growth and reproduction
Crustacean hyperglycemic hormone (CHH)	Peptide	X-organ/sinus gland	Energy metabolism, inhibition of molting
Androgenic gland hormone (AGH)	Protein	Androgenic gland	Sexual differentiation

Four hormone-producing sites are of special importance in crustaceans:

- *The X-organ/sinus gland complex* constitutes the major endocrine control centre and is located in the eyestalk of most higher crustaceans, indicating a close link to extern stimuli, e.g. photoperiod and light intensity. It serves as a storage and release site for several peptide hormones, e.g. molt-inhibiting hormone (MIH), gonad-inhibiting hormone (GIH), crustacean hypoglycemic hormone (CHH) and pigmentary hormones.

- *The Y-organs* are paired and located in the maxillary segments. These glands are the source of the molting hormone ecdysone.

- *The androgenic gland* produces the androgenic gland hormone (AGH) responsible for sexual differentiation and male secondary sexual characteristics. The androgenic gland is lacking in females, where the *ovary* secrets the ovarian hormone, which induces differentiation of the female secondary sexual characteristics.

- *The mandibular organ* releases methyl farnesoate (MF), a terpenoid similar to the juvenile-hormones known from insects, which stimulates the Y-organ and possibly also vitellogenesis.

Figure 1 illustrates the main endocrine centres in crustaceans. Unfortunately, endocrine glands or sites of hormone production in copepods are largely unknown. Table 1 summarises sources and functions of hormones reported in crustaceans.

2.2 Endocrine Regulation of Molting

Crustaceans have to shed their exoskeleton (ecdysis, molting) in order to grow. Levels of the molting hormone 20-hydroxyecdysone fluctuate considerably during the molt cycle, which has four basic stages [29]:

1. **Postecdysis**

   Expansion and hardening of the new exoskeleton, low ecdysteroid hemolymph level

2. **Metecdysis (Intermolt)**

   Tissue growth, storage of food reserves, reproduction (if sexual maturity is reached), low ecdysteroid hemolymph level

3. **Proecdysis**

   Preparation for shedding of old exoskeleton, increase in ecdysteroid hemolymph levels reaching a peak followed by a drastic decrease immediately prior ecdysis

4. **Ecdysis**

   Uptake of water and shedding of old exoskeleton, low ecdysteroid hemolymph level

Ecdysone, the crustacean molting hormone, was first isolated and characterised in the lobster *Jasus lalandei* [30]. The Y-organ primarily produces ecdysone, which, after release to the hemolymph, is rapidly hydroxylated to 20-hydroxyecdysone, the active ecdysteroid in most crustaceans. Moreover, the Y-organ also secretes two other ecdysteroids, i.e. 3-dehydroecdysone and 25-deoxyecdysone, with the latter forming the

precursor of the active ponasterone A (PoA) [31], their role remains to be determined (reviewed in [32]). PoA is the primary circulating ecdysteroid in the premolt stage of the crabs [31]. Advanced analytical techniques, e.g. ELISA, made it possible to quantify ecdysteroid levels also in smaller crustacean species.

For example, changes in 20-HE concentrations through various life-stages of the harpacticoid copepod, *Amphiascus tenuiremis,* and the estuarine amphipod, *Leptocheirus plumulosus,* have been studied recently [33]. In active juveniles of the oceanic copepod *Calanus pacificus,* the variation in ecdysteroid levels relative to the molt cycle phase was similar to that known from decapod crustacean larvae. Ecdysteroid levels measured in field-collected juveniles in diapause (spontaneous state of dormancy) were lower than those in active juveniles [34].

The ecdysone secretion by the Y-organ is under negative control of the molt-inhibiting hormone (MIH), a neuropeptide released from the X-organ/sinus gland (Figure 2). The removal of eyestalks of the fiddler crab, *Uca pugilator*, induced a shortening of the molt cycle. This observation made already in 1905 (cited in [28]) was likely a result of a rapid elevation in the concentration of circulating ecdysteroids due to fact that the Y-organ was not any longer inhibited by MIH. Evidence for the ability of the X-organ/sinus gland to inhibit ecdysteroid secretion by the Y-organ was provided later [35;36]. *In vitro* secretion of ecdysone by crab Y-organs was inhibited when the organs were cultured with conditioned medium that had previously been incubated with explanted sinus glands. The neurotransmitter mediating the release of MIH from the sinus gland is serotonin [35]. In decapods, another neuropeptide with a structure very similar to that of MIH, the crustacean hyperglycemic hormone (CHH), does also inhibit ecdysteroid synthesis [37].

Besides this inhibitory regulation, the Y-organ is under positive control as well. Ecdysone levels in Y-organs cultivated *in vitro* together with mandibular organs were significantly higher compared to those cultured alone. The responsible factor was identified to be a terpenoid produced by the mandibular organ, methyl farnesoate (MF), which is the non-epoxidised form of juvenile hormone-III known from insects (reviewed in [38]). MF stimulates the secretion of ecdysteroids by the Y-organ in particular at the beginning of the proecdysis [39].

In many crustaceans, the overall pattern of growth involves series of instars of similar morphology, followed by a specific molt resulting in substantial morphological changes. For example, in copepods, the sixth molt is linked to a change from the larval to the juvenile morphology. In insects, juvenile hormones control metamorphosis. Their

analogue in crustaceans, MF, is generally believed to play a similar role, but final evidence has not yet provided [37;38].

The synthesis of MF by the mandibular organ is in turn under negative control by another neuropeptid hormone, the mandibular organ-inhibiting hormone (MOIH). This was demonstrated in several studies. The addition of spider crab, *Libinia emarginata*, sinus gland extracts to cultured mandibular organs caused a significant decline in secreted MF *in vitro* [40]. Injection of sinus gland extracts in lobsters, *Homarus americanus*, induced a decline in circulating MF *in vivo* (reviewed in [28]). Subsequent work on this topic resulted in the characterisation and sequencing of MOIH [41;42].

As already mentioned, most of the work elucidating the endocrine control of molting was performed with higher crustaceans, often by eyestalk ablation experiments. How the fluctuation of ecdysteroid levels during molt cycle is regulated in lower crustaceans, which do not have any eyestalk, is still unknown.

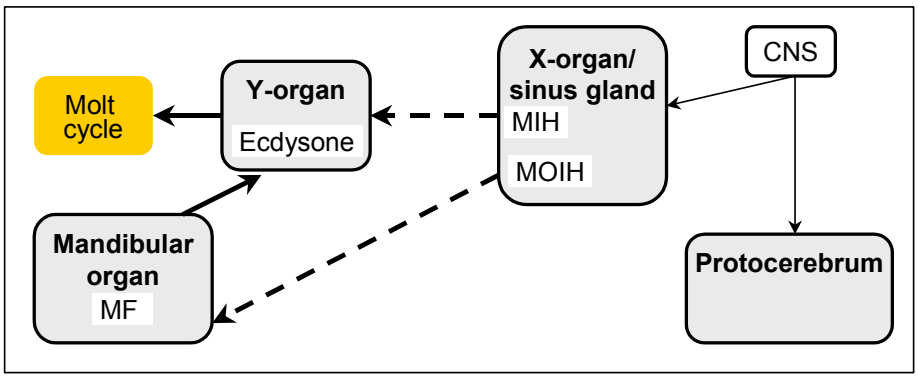


Figure 2 Endocrine control of molting in higher crustaceans (simplified scheme). Solid lines indicate stimulation; dashed lines indicate inhibition. CNS: central nervous system, MOIH: mandibular organ-inhibiting hormone, MIH: molt-inhibiting hormone, MF: methyl farnesoate

2.3 Endocrine Regulation of Sexual Differentiation

Although sex is determined genetically in most crustaceans, many aspects of reproduction, including maturation, maintenance and operation of the gonads, as well as development of secondary sexual characteristics are hormonally regulated [29] (Figure 3). Sexual differentiation in malacostracan crustaceans (e.g. decapods, isopods, amphipods) depends on the presence or absence of a certain protein produced in the

androgenic gland, which is typically associated with the terminal region of the male gamete ducts. This protein, the androgenic gland hormone (AGH) induces male morphogenesis, development of testis and secondary male sexual characteristics. In females, the primordial androgenic gland does not develop and the ovaries auto-differentiate spontaneously, i.e. in the absence of AGH [43].

The role of the androgenic gland for sexual differentiation was elucidated in ablation and implantation experiments. In male prawns, *Macrobrachium rosenbergii*, the ablation of the androgenic gland induced feminisation including the initiation of oogenesis and development of oviducts and female gonopores [44]. Re-implantation of the androgenic gland revoked these effects. In penaed shrimps, *Penaeus indicus*, and isopods, *Armadillidum vulgare,* ablation of the androgenic gland caused demasculinisation [45]. On the other hand, the implantation of the androgenic gland into females caused masculinisation in *M. rosenbergii* [46] and functional sex reversal in *A. vulgare* [47].

2.4 Endocrine Regulation of Reproduction

The endocrine control of crustacean reproduction has been investigated in a wide variety of species (for reviews see [21;28]). In males, peptide hormones are involved in the maintenance of the germinative tract via the regulation of the activity of the androgenic gland. A stimulating neurohormone was isolated from the brain and thoracic ganglia, the gonad-stimulating hormone (GSH). The X-organ/sinus gland is the source of the gonad-inhibiting hormone (GIH), which was demonstrated by eyestalk removal in immature crabs resulting in precocious gonad development [21].

The hormonal regulation of female reproduction has been reviewed in detail [48]. The synthesis of vitellogenin, the precursor of vitellin, the major yolk protein of mature crustacean eggs, is under subtle hormonal control. Several tissues, e.g. the ovary, hepatopancreas and subepidermal adipose tissue have been postulated as the sites of vitellogenesis [49]. GSH has a stimulatory effect, whereas GIH inhibits the synthesis of vitellogenin and its uptake by the fat body. The latter is therefore also called vitellogenesis-inhibiting hormone (VIH) [43;48]. In some amphipods, vitellogenesis is under positive control of the vitellogenin-stimulating ovarian hormone (VSOH), which seems to play a similar role as 17β-estradiol in egg laying vertebrates [43]. In an isopod, *A. vulgare*, vitellogenesis was shown to be under negative control of AGH by ablation of the androgenic gland from males, which initiated synthesis of vitellogenin by the fat body, whereas, implantation of the androgenic gland to females inhibited vitellogenin

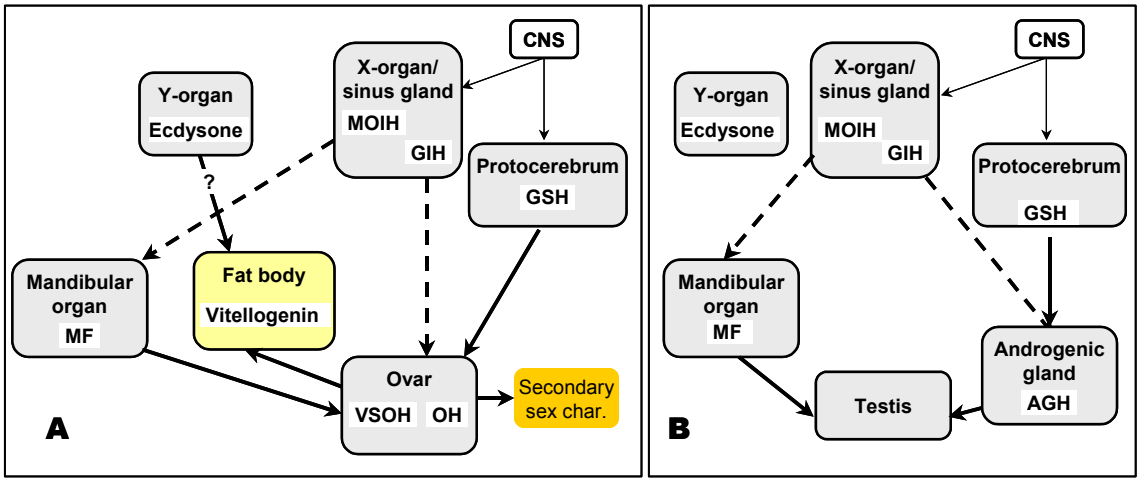


Figure 3 Endocrine control of sexual differentiation and reproduction in a female (A) and a male (B) crustacean (simplified scheme). Solid lines indicate stimulation; dashed lines indicate inhibition. CNS: central nervous system, MOIH: mandibular organ-inhibiting hormone, GIH: gonad-inhibiting hormone, GSH: gonad-stimulating hormone; MF: methyl farnesoate, AGH: androgenic gland hormone, VSOH: vitellogenesis-stimulating ovarian hormone, OH: ovarian hormone

synthesis [50]. Thus, vitellogenesis in crustaceans is under negative regulatory control by an androgen, whereas in oviparous vertebrates estradiol positively controls vitellogenesis.

Elevated levels of MF found in the hemolymph of vitellogenic females as well as reproductively active males revealed that also this hormone is important for reproduction [51]. MF stimulates ovarian maturation in crayfish [52]. However, the specific role of MF in crustacean reproduction is unclear [28]. Several studies with higher crustaceans indicated, that also ecdysteroids are involved in reproduction, vitellogenesis, and embryogenesis (reviewed in [31]). Recently, this was also shown for lower crustaceans, e.g. copepods and amphipods [33]. However, the specific function of ecdysteroids for reproduction remains to be determined and may vary among taxa [28].

Because of the complexity of vitellogenesis and the general shortage of fundamental knowledge of crustacean endocrinology, the induction of vitellogenin has not been used as a biomarker of endocrine disruption to the same extent as the up-regulation of this egg yolk protein in fish [53]. However, a few studies reported induction of vitellin or vitellin-like proteins after exposure to estrogenic compounds in grass shrimps, *Palaemonetes pugio* [54] and barnacles, *Balanus amphititre* [55]. Very recently, vitellin from an estuarine mysid, *Neomysis integer*, was purified and characterised as a first step to develop immunoassays to study vitellogenesis in mysids [56]. Volz and Chandler

[57] developed an ELISA capable of detecting vitellogenin in copepods and other crustaceans at concentrations as low as 2 ng/ml, which may be applied in evaluating endocrine activity of environmental toxicants.

2.5 Vertebrate-Type Steroid Hormones

In Crustaceans, estradiol and estrone were identified in 1978 by Jeng et al. [58]. Subsequent studies demonstrated the presence of these hormones and progesterone in the ovary, hepatopancreas and the hemolymph [59-62]. Testosterone has been isolated from and identified in serum and testes of lobsters [63]. For more detailed information on the presence of vertebrate-type hormones including steroids in crustaceans see the review by Fingerman et al. [64]. Several studies revealed a notable capability of crustaceans, e.g. the cladoceran *Daphnia magna* and mysid shrimps, to metabolise testosterone [65-68]. Recently, we provided first evidence for the existence of a specific testosterone binding-site in cytosolic extracts of whole body homogenates of the amphipod *Hyalella azteca* using a radio-receptor assay (Lutz and Wollenberger, unpublished data). Altogether, there are strong indications that vertebrate-type steroids may function as hormones in crustaceans, however, their precise physiological role and mechanisms of action remain to be elucidated.

3 Materials and Methods

3.1 Selection of Test Species

In the initial phase of this project, the chronic toxicity of antibiotics towards crustaceans was investigated **[1]** using the well-established *Daphnia magna* reproduction test [27]. During the course of the project, special emphasis was put on potential EDCs. Numerous studies describe effects of EDCs like alkylphenols, phthalates and vertebrate steroid hormones on *D. magna,* e.g. [69-73]. Like many other cladocerans, daphnids are parthogenetic under favourable environmental conditions. In some of those studies, a switch to sexual reproduction was induced by for example reduction in food supply, high population density [74] or shortening of the photoperiod [75]. This implies the exposure of test animals under sub-optimal conditions and might therefore influence their sensitivity towards toxic stress and thus impact test results. Neither working with an asexually reproducing organism nor exposure of *D. magna* under sub-optimal conditions to prevent parthogenesis were considered an appropriate approach for developing a test for detecting sublethal effects of potential endocrine disrupters. Hence, it was decided to look for small crustaceans with sexual reproduction as alternatives of *D. magna.* The calanoid copepod *Acartia tonsa* and the harpacticoid copepod *Nitocra spinipes* were selected, their cultivation in the laboratory optimised and sensitive sublethal endpoints that may be responsive to EDCs identified in sublethal toxicity tests with these species.

3.2 Development of a Fully Defined Marine Medium

To my knowledge, no fully defined marine medium developed specifically for cultivation of *A. tonsa* and the organism it feeds on, the cryptophycean alga *Rhodomonas salina*, and toxicity testing was available when I had started my Ph.D. work. In particular, when working with potential endocrine disrupters, which can affect organisms at extremely low concentrations, it is of greatest importance to avoid any contamination of the medium, which might confound the test results. By now, mainly natural seawater was used for toxicity testing with marine crustaceans, whose composition can vary from laboratory to laboratory or day to day in respect to the site and time of sampling. Consequently, contamination with chemicals that might contribute to the overall effect observed in the toxicity test could neither be excluded nor quantified. Thus, there was a need for a fully defined marine medium suitable for cultivation of the test organism, its food as well for performing toxicity tests.

In this study, a fully defined media was developed allowing the cultivation of *A. tonsa* and *R. salina* without problems for eight month **[2]**. Meanwhile this medium has been in use for more than five years in our laboratory and was successfully applied in various studies **[3;4;6].** Moreover, this newly developed medium should be considered for cultivation of other marine organisms.

To check the sensitivity of *A. tonsa* towards chemicals when cultivated in the new medium, 48-h acute toxicity tests according to ISO 14669 [76] were performed with bisphenol A, nonylphenol and the two reference compounds, 3,5 DCP and potassium dichromate. The determined LC_{50} of 16 mg/l for potassium dichromate and the corresponding 95% confidence interval of 13 to 21 mg/l were in-line with results of a previous interlaboratory comparison **[2].** The advantage of using fully defined marine media was also recognised by the OECD working group in the revised version of the proposal for a new OECD Draft Guideline for Testing of Chemicals - Calanoid Copepod Development and Reproduction Test with *Acartia tonsa* **[8].**

3.3 Copepods

3.3.1 Overview of Copepod Biology

The *Copepoda* form a subclass of the class *Crustacea*. Approximately 12,000 copepod species have been described until 1993 [29]. *Calanoida* and *Harpacticoida* are two out of ten copepod orders. The name copepod originates from the Greek words *kope* (oar) and *podos* (foot), and refers to the flat, laminar swimming legs of the animals [77]. Copepods are microscopic, primarily marine animals and represent important prey items for larvae of many fish and larger invertebrates and are increasingly used as a live food source in aquaculture.

Copepods are sexually dimorphic; males are usually smaller than females. During copulation, the males transfer sperm to the females by placing a spermatophore close to the opening of the egg duct of the female. After mating, females can store the sperm and therefore, a single copulation enables females to produce fertilised eggs for a longer time, in some species throughout the entire reproductive period. In calanoids, eggs are released singly into the water, but in most other copepods, e.g. harpacticoids, eggs are deposited by the female in one or two egg sacs (ovisacs) and brooded until the nauplii hatch. During development, copepods pass through six naupliar and five copepodite stages before turning into adults. The copepodites have the adult shape, while the nauplii are less differentiated and almost circular. Newly hatched, the unsegmented first nauplius stage already possesses three pairs of appendages. At each successive molt,

these appendages develop further setae or segments. Moreover, rudimentary forms of other appendages and additional somites (body segments) develop during the nauplius stages. The first copepodite stage displays the general adult features, but the abdomen is still unsegmented. Segmentation of the abdomen takes place and limbs are formed with each subsequent molt. The adult copepod has got ten somites with six pairs of appendages [29].

3.3.2 *Acartia tonsa*

Acartia tonsa Dana belongs to the calanoid copepods, tolerates salinities between 10 and 35 ‰ and is a very common species in Danish coastal waters. Species of the genus *Acartia* are widely distributed in brackish and coastal waters around the world and often dominating in the zooplankton. They feed on planktonic microalgea and are thus ecologically important links between primary producers and organisms of higher trophic levels in marine food webs.

A detailed description of *A. tonsa* and a protocol for its cultivation are given in **[8]**. Depending on temperature (15 to 20 °C), *A. tonsa* reaches the adult stage after 9 to 20 days [77]. In the laboratory under optimised conditions, a female can produce up to 60 eggs per day, which hatch after 24 hours. The body length of females is about 1.0 mm, while that of males is only around 0.8 mm. Males and females are easily to distinguish from each other (Figure 4). *A. tonsa* has been used in ecotoxicological tests for many years [78]. An International Standard (ISO) for acute toxicity testing exists [76].

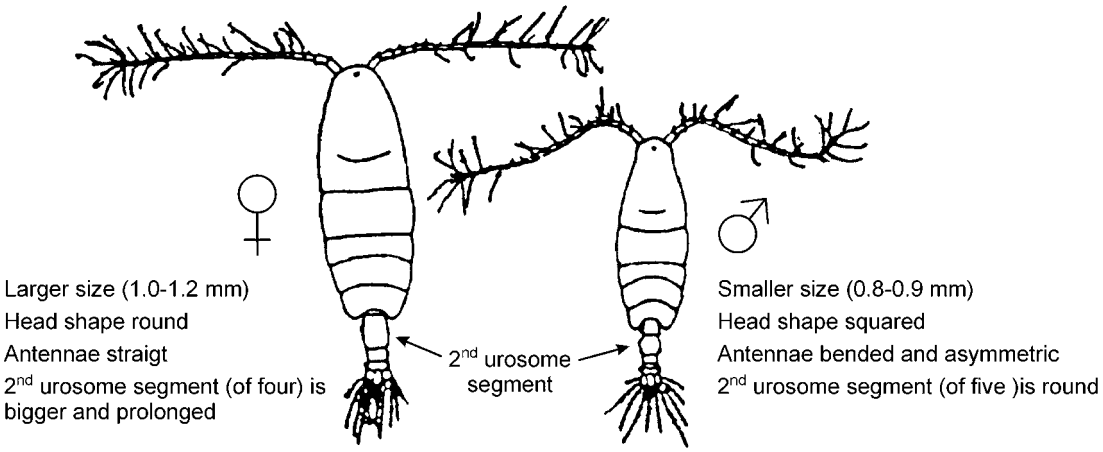


Figure 4 Main secondary sex characteristics of a generalised calanoid copepod (the specification of size is related to *Acartia tonsa*)

3.3.3 *Nitocra spinipes*

Nitocra spinipes Boeck is a deposit-feeding harpacticoid copepod, which is a common component of the benthic meiofauna in shallow coastal waters of many regions around the world. It tolerates salinities between 0 and 35 ‰. Females have a body length of about 0.75 mm and are generally bigger than males (0.45-0.56 mm). At 22 °C, *N. spinipes* reaches sexual maturity within 10-12 days; the generation time is 16-18 d. Females develop single egg sacs (Figure 5) and can produce at least six broods during their life [79]. *N. spinipes* has been used in ecotoxicological tests since 1975 [80]. An International Standard (ISO) for acute toxicity testing exists [76].

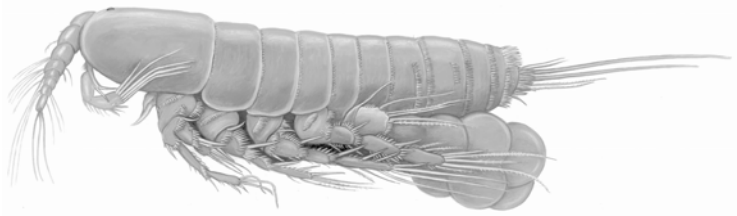


Figure 5 Ovigerous *Nitocra spinipes* female (Drawing by Göte Göransson)

3.4 Copepods as Test Species for (Sub)chronic Toxicity Testing

General criteria for the selection of invertebrate species suitable for identifying toxic effects related to endocrine disruption in the environment were given by Ingersoll et al. [22] and include primary mode of reproduction, suitability for culturing in the laboratory, generation time, size of organism, knowledge on endocrinology, and availability of standard methods. The copepod species used in this study reproduce sexually and endpoints like fecundity or sex ratio are applicable. Laboratory cultures are well established and the short generation time allows conducting full life-cycle and trans-generational exposures. On the other hand, organisms with a short generation time are typically small. The size of an organism is important not only for practical reasons, e.g. required space, amount of food, but also for determining concentrations of hormones in the tissue. For instance, the dry weight of an adult *A. tonsa* individual is only 5 µg (**this study**, unpublished data). This implies that it would not be possible to get enough tissue for chemical analysis when working with *A. tonsa* and, therefore, the determination of hormone concentrations in tissue samples was excluded from the scope of this study from the first. Basic knowledge on the endocrine system of the test

organism is important for the identification of endpoints related to endocrine controlled processes, which can be expected to be susceptible towards endocrine disruption. Though our knowledge on crustacean endocrinology is limited, the crustacean and insect hormone systems are the best understood among invertebrate phyla and some information on the endocrine regulation of e.g. molting and reproduction is available (chapter 2).

The characteristics and biological attributes given in Table 2 facilitate the measurement of survival, hatching success, larval development ratio, body length, sexual maturation, egg production, egg diameter and sex ratio. These endpoints can be categorised as follows: General toxicity (mortality), endpoints related to growth and molting as well as endpoints related to sexual differentiation and reproduction (Figure 6).

Table 2 Fundamental characteristics of *Acartia tonsa* and *Nitocra spinipes* related to their suitability as test species in ecotoxicology

Acartia tonsa	***Nitocra spinipes***
• Pelagic	• Benthic
• Easily to culture under laboratory conditions	• Easily to culture under laboratory conditions
• Non-cannibalistic	• Non-cannibalistic
• Sexual reproduction	• Sexual reproduction
• Continuous release of individual eggs (up to 60 per female and day)	• Single egg sac
• Nauplii and copepodites are easily to distinguish from each other	• Nauplii and copepodites are easily to distinguish from each other
• Visual sex determination feasible	• Visual sex determination feasible
• Generation time 14 d (at 20 °C)	• Generation time 17-20 d (at 20 °C)
• Size 0.8-1.0 mm	• Size 0.45-0.75 mm
• ISO Standard available for acute toxicity testing	• ISO Standard available for acute toxicity testing

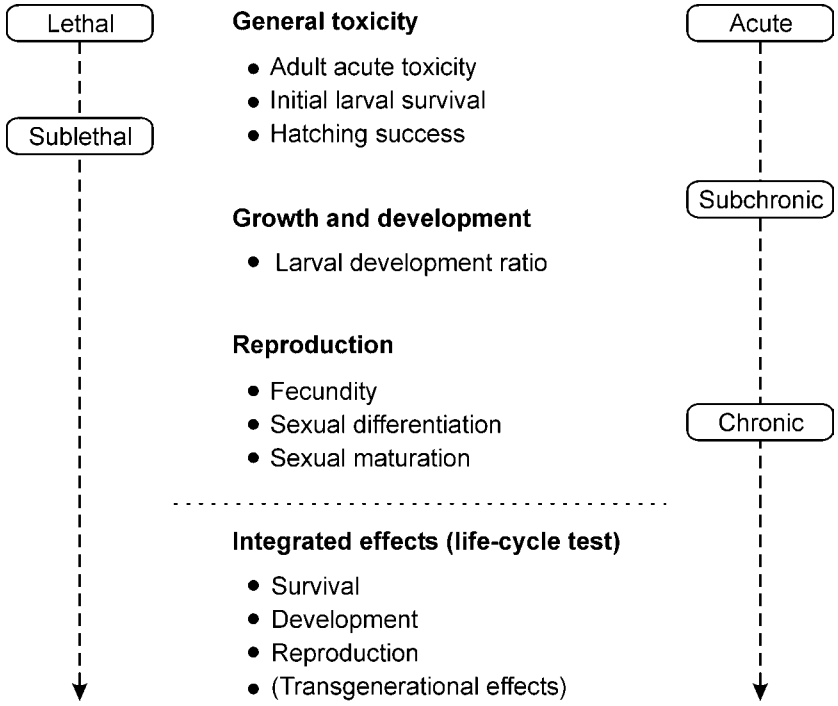
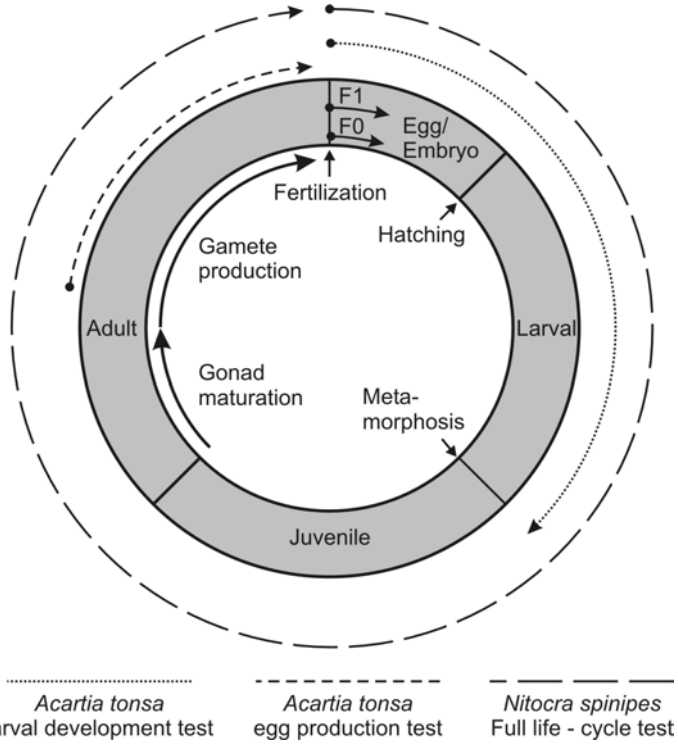


Figure 6 Lethal and sublethal endpoints related to general toxicity, growth and development, and reproduction measurable in acute, subchronic and chronic toxicity tests using the calanoid copepod *Acartia tonsa* and the harpacticoid copepod *Nitocra spinipes*

Lethal effects are studied in short-term ecotoxicity tests, where the exposure time is short compared to the generation time of the organism. Sublethal effects as for example inhibited larval development or decreased reproductive success, are studied in subchronic and chronic tests. In chronic toxicity tests, organisms are exposed a substantial period of their life cycle, while in subchronic tests, exposure time is in-between that of acute and chronic tests. Full life-cycle tests cover all life stages, which may not be equally sensitive to a given compound. They can thus include embryo-larval stages as well as periods of gonad development and reproduction. Moreover, exposures can be extended over more than one generation to include some or all key life stages in the next generation (Figure 7). Since the sensitivity of certain developmental stages and critical periods of endocrine function often are not known, full life-cycle tests are considered the best way to study EDCs with regard to their environmental risk [22].

Full life-cycle test with various copepod species, for example *Tisbe battagliai* [81;82], have been used in a number of studies with EDCs. In the present study, selected model compounds (polybrominated diphenyl ethers and synthetic musks) were studied in a full life-cycle test with *N. spinipes* **[5;7]**.



Figur 7: Crucial developmental stages of crustaceans, which might be sensitive towards endocrine disrupters, in a life-cycle context. A partial life-cycle test covers a certain stage of development, whereas a full life-cycle test covers all stages of the F0 generation and might be extended to some or all stages of the F1 generation in a trans-generational exposure. The methods applied in this study are plotted in relation to the life-cycle of the test organism. (Modified from [22])

The primary objective of this dissertation was, however, the identification of sensitive endpoints applicable to screen chemicals for sublethal effects using *A. tonsa*. Such screening tests should be easily to perform, be accomplished within a reasonable timeframe and give a reliable indication of chronic toxicity of the tested compound. Hence, in this study endpoints like larval development ratio and egg production were investigated in detail in subchronic exposure experiments, so-called partial life-cycle tests, with *A. tonsa*. In a follow-up project, these endpoints were implemented in a trans-generational full life-cycle test covering the interval from hatching of the F0 generation to the appearance of copepodites of the F1 generation **[8]**.

3.5 Endpoints Investigated in *A. tonsa*

The methods applied including the experimental details are given in the papers included in the annex **[2-8]** and hence, summarised in the following only briefly. Adult acute toxicity tests were performed prior to subchronic and chronic testing of the chemicals in order to define the concentrations to be tested. Generally, the LC_{10} values obtained in the acute toxicity test were set the upper limit of the concentration range for the subchronic and chronic tests. *N. spinipes* 96-h acute toxicity tests as well as *A. tonsa* 48-h acute toxicity tests were performed according to ISO 14669 [76].

3.5.1 Larval Development Ratio

Starting point for the larval development tests was a study published in 1997 in which the authors reported that the known endocrine-disrupting chemical TBT was a highly potent inhibitor of the development of *A. tonsa* larvae with an EC_{50} 160 times lower than the LC_{50} value obtained in an acute toxicity test with adults. In contrast, linear alkylbenzene sulfonate, which is not associated with endocrine disruption, did not affect larval development at sublethal concentrations [78]. The development of copepods includes several molts and one metamorphosis from the nauplius to the copepodite morphology. Both processes, molting and metamorphosis, are regulated by ecdysteroids. Furthermore, the metamorphosis is presumably controlled by MF, similar the juvenile hormones regulating metamorphosis in insects (chapter 2). For this reason, it was studied, how far a test based on measuring the percentage of test animals that have turned from nauplii into copepodites in a certain time period, would be suitable to screen potential EDCs for (sub)chronic toxicity.

The larval development test covers the period of development from hatching to the first copepodite stages (Figure 7 and 8). The easily detectable change from the nauplius to the copepodite morphology was utilised as endpoint. At the time, when about 50% of organisms in the control had reached a copepodite stage, which takes 5 days at 20 °C, the larval development ratio (LDR), expressed as the percentage of test organisms that have turned into copepodites (number of copepodites/[number of nauplii + copepodites]), was recorded for each replicate of the treated groups and compared with that of the control. Choosing the time when attaining LDR = 50% in the control as time to determine LDR in the exposed groups enables the detection of both stimulation as well as inhibition of larval development.

Experiments were started by adding a known number of eggs (30-40) produced by the copepod stock culture within a period of 24 h to 100 ml glass beakers containing 80 ml of working test solution. The test included six concentration levels in a geometric

concentration series. Twelve replicates were used for controls and four for each test concentration. The exposure was conducted at 20 ± 0.5 °C and under a light regime of 16 h light to 8 h dark and semi-static conditions with feeding of micro-algae (*R. salina)*. To ensure that the experiments were finished when the LDR in the control was approximately 50%, the number of nauplii and copepodites was determined in one control replicate after exactly 5 days. If this control contained less than 50% copepodites, the test was allowed to run for another hour, after which an additional control replicate was studied, etc. At the end of the test, unhatched eggs, nauplii and copepodites were counted in all replicates. EC values for the inhibition of the LDR were calculated. Additionally, hatching success and larval mortality were recorded.

It is worth noting that a constant temperature is essential in conducting the larval development test, in particular it is absolutely necessary to prevent any temperature gradient between the individual beakers used in the test. Minor deviations in temperature delay or accelerate larval development significantly (unpublished data).

In the course of this study, inhibition of the development of *A. tonsa* larvae has proven to be the best suitable parameter for screening purposes. Larval development tests were performed with in total 38 compounds, including natural vertebrate and invertebrate hormones, synthetic hormones, industrial chemicals, pesticides, brominated flame retardants, synthetic musks and a set of reference compounds (Table 3).

3.5.2 Sex Ratio

In preliminary experiments, the parameter sex ratio (number of females divided by the total number of animals) was investigated. Animals were exposed during the period of development from egg to adults under the same temperature and light conditions as described above for the larval development test. The exposure lasted 12 to 14 days. The sex ratio in the controls varied between about 0.5 and 0.8. Due to the high variability in the sex ratios seen in control groups and the rather time-consuming and labour-intensive method, sex ratio was not considered further a suitable endpoint for screening purposes. However, for the time being it is envisaged one of the endpoints in the full life-cycle test with *A. tonsa* **[8]**.

3.5.3 Egg Production

Some copepods like *A. tonsa* produce eggs continuously from sexual maturation to the end of their life span. Since *A. tonsa* releases eggs one by one to the water, it is relatively easy to count the number of eggs released per female during a certain period of time and use this parameter as a reproductive endpoint. From previous studies with *A. tonsa* it was noted that egg production during the first three days of sexual maturity

rises to a constant level, which is the production capacity of the animals with the available food resources [83]. Under the laboratory conditions chosen in the present study, egg production started when the copepods were about 10 days old. The number of eggs produced per female within 24 h was counted from day 14 to 15 and again from day 16 to 17, i.e. during the period of maximum egg production capacity.

Test organisms aged 11 days were exposed for 6 days and the number of eggs produced per female during a period of 24 h was determined on day 4 and 6. Thus, prior to measuring egg production the animals were pre-exposed for 72 and 120 h, respectively. A geometric series comprising six different concentrations with 4 replicates per concentration was tested. Each replicate contained 80 ml of test medium and 10 animals, which were sexed after completion of the test on day 17 in order to determine the number of females present in the respective replicate. Sex determination was carried out at the end of the test since it was impossible to sex living *A. tonsa* without stressing or damaging them. The exposure was conducted under semi-static conditions, at 20 ± 0.5 °C with a 16 h light to 8 h dark rhythm. Organisms were fed with micro-algae (*R. salina*). The test vessels consist of 100 ml beakers with an inner chamber that holds the test animals. The bottom of this chamber is made of a stainless steel sieve of 180 µm mesh size. *A. tonsa* eggs have an average diameter of 80 µm (unpublished data) and can thus pass the sieve and sink to the bottom of the beaker. After the observation period of 24 h, the inner chambers containing the animals were carefully transferred to other beakers containing fresh test solution and the remaining eggs were counted. The number of eggs produced per female of the exposed groups was compared to those of the control groups and effect concentrations (EC_X, LOEC, NOEC) determined.

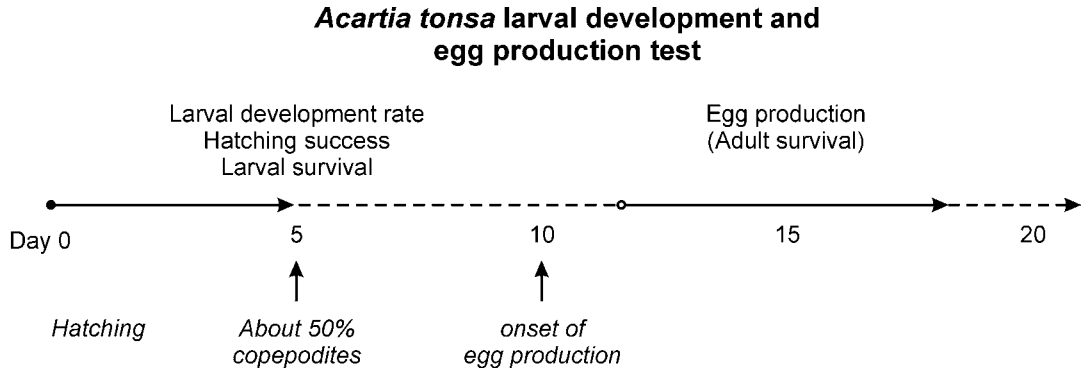


Figure 8 Endpoints related to development and reproduction recorded in the larval development and the egg production test with the calanoid copepod *Acartia tonsa* (above the line); approximate age in days and crucial events in the life-cycle (below the line)

3.6 Full Life-Cycle Test with *N. spinipes*

The work with *N. spinipes* was performed in close co-operation with Magnus Breitholtz at the Institute of Applied Environmental Research (ITM), Stockholm University, Sweden. The full life-cycle test with *N. spinipes* is described in detail in **[5;7]**. Nauplii less than 36 hours old were randomly allocated to vials containing 5 ml of brackish water. Each vial, with 10-15 nauplii, was then randomly allocated to groups, of which each comprises 8 replicates. Thereafter, 5 ml of a test solution containing the chemical to be studied in a certain concentration were added to the vials belonging to a particular group. Every second day, copepods were fed with a feed suspension prepared from commercial salmon feed as well as 70% of the test solutions renewed. On day 6-8, the first endpoint, the LDR, was recorded as the percentage of copepodites among all offspring divided by the total number of juveniles at the beginning of the experiment. At this time about 50% of the individuals had normally reached the copepodite stage. Mortality rates were determined by counting the living organisms on day 6-8. On day 15-18, ovigerous females started to appear and were then individually transferred to vials containing fresh test solution. The exposures were generally terminated on day 18. However, individually isolated ovigerous copepods were left in exposure to hatch their eggs for additional four days. Released nauplii and aborted egg sacs were counted. Remaining copepods were fixed in formaldehyde (4%), sexed and examined for morphological malformations. If the average control mortality plus handling losses exceeded 20% on day 6-8 or 30% on day 18-22, the experiments were considered not valid. Figure 9 shows the endpoints, which are related to larval development, i.e. LDR, as well as to later developmental stages and reproduction, i.e. mortality rate, time to release of first brood, sex ratio, the fraction of ovigerous females among all females and the number of nauplii per ovigerous female. A modified Euler-Lotka equation **[5]** was used to calculate a population level endpoint, the intrinsic rate of natural increase (r_m) from these individual life-table endpoints.

3.7 Ecdysteroid Receptor-Based *in vitro* Screening Assay

The work with the *Drosophila malanogaster* B_{II} cell *in vitro* assay was performed in close co-operation with Laurence Dinan at the Department of Biological Sciences, University of Exeter, U.K. The B_{II} test was developed by Clement and Dinan in 1993 [84] and is described in **[5;6]**. The assay was performed with four synthetic musks **[5]**, six brominated flame retardants **[6]** as well as vinclozolin and fenoxycarb (**this study**, unpublished data).

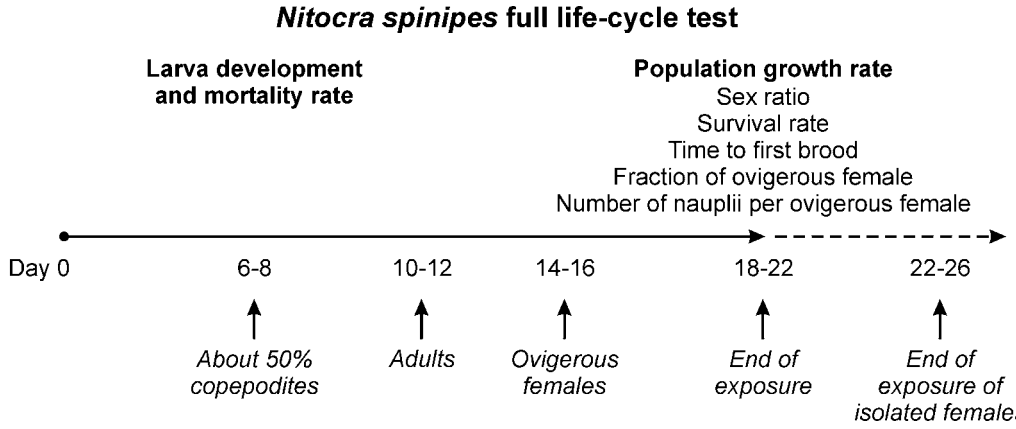


Figure 9 Endpoints related to development, reproduction and population growth recorded in the full life-cycle test with the harpacticoid copepod *Nitocra spinipes* (above the line). Approximate age in days at crucial events in the life-cycle test (below the line)

The B_{II} screening assay for detection of ecdysteroid agonists and antagonists is ideally suited to the evaluation of environmental contaminants as potential EDCs [85] and proven to be reliable, simple and robust [86]. The B_{II} bioassay is based on the ecdysteroid response of the *Drosophila malanogaster* B_{II} cell-line [84]. The bioassay is performed by growing the cells in the wells of microtitre plates and the response can be measured turbidimetrically. In the presence of 20-HE or a compound with ecdysteroid agonist activity, these cells form phagocytotic clumps with concomitant increase in cell size and reduction in cell density. The assay is sensitive, giving a full agonist response with 5 ng 20-HE equivalents per well and a detectable response with 0.5 ng 20-HE equivalents [86]. By addition of a known amount of 20-HE per well, the test can be modified to test for the presence of ecdysteroid receptor antagonists.

When testing for endocrine disruption *in vivo*, it is important to remember that many effects on endocrine regulated processes can occur via other specific mechanisms or due to general toxicity. As a complementary method to *in vivo* studies, the B_{II} assay can contribute to a better understanding of the mechanisms behind effects observed in *in vivo* tests.

3.8 Acute-to-Chronic Ratios

Another approach to distinguish effects caused by specific modes of toxic action from those related to general toxicity is the acute-to-chronic ratio (ACR). The ACR is defined as the ratio of a chemicals acute toxicity expressed as e.g. LC_{50} and its chronic toxicity expressed as e.g. LOEC or EC_{50} and varies strongly depending on the mode of action

[87]. The ACR is typically below 10 for pure baseline toxicants and can be much higher if the mode of toxic action in the chronic test is different from that in the acute test [88]. It is important to note that only ACR values based on the same chronic toxicity parameter (e.g. either LOEC or EC_{50}) may be compared and hence, published data should be interpreted with care taking this fundamental assumption into account. In risk assessment, a factor of 10 is often used to extrapolate from acute effect concentrations to chronic effect values [89]. Thus, this factor is assumed to reflect differences between acute and chronic toxicity values. In this study, an ACR exceeding 10 was therefore considered as indication for a specific mode of toxic action. ACRs were calculated using LC_{50} from adult acute toxicity test with *A. tonsa* and EC_{50} derived from the larval development test. ACRs derived from tests with *N. spinipes* were calculated using LC_{50} from acute toxicity test and LOEC from full life-cycle tests. This implies that ACRs derived from *A. tonsa* tests and *N. spinipes* tests cannot readily be compared.

3.9 Test Compounds

Tabel 3 provides an overview of the compounds investigated in this study, their known endocrine effects as well as the applied test methods.

Table 3 Chemical name, known endocrine effects of and tests performed with the 38 investigated compounds. ✓/- indicate whether a particular test was carried out or not. LDR means larval development ratio

Substance group/compound	Primary endocrine effects	B_{II} cell in vitro assay	Tests with *A. tonsa*		Test with *N. spinipes*
			LDR	Egg prod.	Full life-cycle
Natural vertebrate hormones					
17β-Estradiol, E2	Estrogen	-	✓	✓	-
Estrone, E1	Estrogen	-	✓	✓	-
Progesterone	Estrogen	-	✓	✓	-
Testosterone	Androgen	-	✓	✓	-
Methyltestosterone	Androgen	-	✓	✓	-
Natural invertebrate hormones					
20-Hydroxyecdysone	Ecdysteroid	-	✓	✓	-
Ponasterone A	Ecdysteroid	-	✓	-	-
Juvenile hormone-III	Juvenile hormone	-	✓	✓	-
Pharmaceuticals/synthetic hormones		-			
17α-Ethinylestradiol, EE2	Estrogenic	-	✓	✓	-
Diethylstilbestrol, DES	Estrogenic	-	✓	✓	-
ICI 182, 780	Anti-estrogenic	-	✓	-	-
Cyproterone acetate	Anti-androgenic	-	✓	-	-
Flutamide	Anti-androgenic	-	✓	✓	-
Hydroxyflutamide	Anti-androgenic	-	✓	-	-
Tamoxifen	Anti-estrogenic	-	✓	-	-
Industrial chemicals		-			
4-Octylphenol	Estrogenic	-	✓	-	-
Nonylphenol ethoxylate, NPEO[a)]	Estrogenic	-	✓	✓	-
Nonylphenol acetat[b)]	Not known	-	✓	-	-
Bisphenol A	Estrogenic	-	✓	✓	-
Diethyl phthalate	Estrogenic	-	✓	-	-
Pesticides		-			
Vinclozolin	Anti-androgenic	✓	✓	-	-
Methoprene	Juvenile hormone antagonist	-	✓	-	-

Table 3. (continued)

Fenoxycarb	Juvenile hormone mimic	✓	✓	-	-
p,p'-DDE	Anti-androgen	-	✓	-	-
Synthetic Musks					
Musk ketone, MK (nitro musk)	Estrogenic	✓	✓	-	✓
Galaxolide, HHCB (polycyclic musk)	Estrogenic, anti-estrogenic	✓	✓	-	✓
Tonalide, AHTN (polycyclic musk)	Estrogenic, anti-estrogenic	✓	✓	-	✓
Celestolide, ADBI (polycyclic musk)	Estrogenic	✓	✓	-	✓
Brominated flame retardants					
2,4,6-Tribromophenol, TBP	Thyroid hormone-like	✓	✓	-	-
Tetrabromobisphenol A, TBBPA	Thyroid hormone-like	✓	✓	✓	-
2,4,4'-Tribromodiphenyl ether BDE-28	Estrogenic	✓	✓	-	-
2,2'4,4'-Tetrabromodiphenyl ether, BDE-47	Estrogenic	✓	✓		✓
2,2'4,4',5-Pentabromodiphenyl ether, BDE-99	Estrogenic	✓	✓		✓
2,2'4,4',6-Pentabromodiphenyl ether, BDE-100	Estrogenic	✓	✓		✓
Reference compounds					
Potassium dichromate	-	-	✓	✓	-
3,5-Dichlorophenol	-	-	✓	✓	-
3,4-Dichloroaniline	-	-	✓	✓	-
Acetone (carrier solvent)	-	-	✓	-	-

[a] Technical mixture. The average number of ethoxylate groups is 10

[b] Metabolite of nonylphenol ethoxylates

4 Effects of Potential Endocrine Disrupters on Crustaceans

4.1 Introduction

Although endocrine disruption in invertebrates has not been studied as extensive as in vertebrates, in particular in fish, numerous reports on the effects of EDCs on crustaceans have been published since the beginning of the nineties, e.g. [66;90;91]. By far most of these investigations were laboratory studies but a few field studies do also exist [92;93]. The majority of early reports on endocrine effects in crustaceans dealt with compounds for which estrogenic or anti-estrogenic effects had already been detected in vertebrates, e.g. alkylphenols, BPA, phthalates, organochlorine pesticides and natural as well as synthetic hormones. The results of these investigations were in part inconsistent and showed that some crustacean groups may be affected by exposure to EDCs, but other may not and, therefore, extrapolation of results from one crustacean group to another is problematic. Effects of potential EDCs in crustaceans may be other than those anticipated by knowledge of their actions in vertebrates. It is known that some processes regulated by estrogens in vertebrates are controlled by androgens in crustaceans, e.g. sexual differentiation (chapter 2). Therefore, androgens and anti-androgens were included in studies on endocrine disruption in invertebrates as well. Another frequently studied group of substances comprises compounds, which affect specifically the hormonal system of arthropods. To this group belong hormones unique in arthropods such as juvenile hormones and ecdysteroids as well as chemicals that have been purposely synthesised to disrupt the endocrine system of a number of insects to aid their control. These so-called insect growth regulators represent third generation insecticides and were developed to intentionally interact with the hormonal system of arthropods, acting as ecdysone agonists or antagonists or as juvenile hormone analogues. In most studies, existing ecotoxicological test protocols were applied sometimes modified or extended in order to investigate endpoints related to processes regulated by hormones as growth, molting, sexual maturation and offspring production. These endpoints were for example considered for developing life-cycle tests with copepods and mysid shrimps [**5;7**;82;94;95]. Only recently, efforts have been undertaken to develop new methods using endpoints like DNA-response, steroid hormone levels and steroid metabolism as biomarkers for endocrine disruption [57;67;96;97]. A few receptor binding assays are available such as the B_{II} test for identification of compounds with (anti)ecdysteroid activity [86].

In this thesis, a comprehensive literature survey on endocrine disruption in crustaceans was conducted, which has been summarised in Table 4. In principle, endocrine

disruption in crustaceans cannot be considered irrespective of research on endocrine disruption in other invertebrates. In particular, studies on insects are of fundamental importance for the interpretation of the effects seen in crustaceans. Nevertheless, the information given in Table 4 was confined strictly to crustaceans.

From the author's point of view, the Table 4 summarises the results of the most relevant publications available by September 2004. It comprises about 100 studies, which were organised with reference to the organism groups and contains information on the compounds studied, the effects observed and the effective concentrations. From the large number of cited references it becomes obvious that it would be impossible to deal with all studies within the framework of this synopsis. That is why those interested in more detailed information are referred to recent reviews [22;98-101] but it should be noted that these reviews are not as complete and up-to-date as that given here.

Many of the compounds studied by other authors were incorporated in the investigations conducted during this Ph.D. work (Table 3). The results of these experiments were discussed in detail in the publications enclosed in the annex, and hence, will not be repeated below. An overview of important so far unpublished results is given in Table 5. In the course of this study it has become apparent that the larval development ratio (LDR) was the most suitable endpoint for screening of potential EDCs. For this reason, the following discussion focuses primarily on literature referring to endpoints related to molting and development.

4.2 Ecdysteroids, Juvenile Hormones and Insect Growth Regulators

4.2.1 Ecdysteroids

Ecdysteroids, the molting hormones in arthropods, are important as reference compounds for evaluating effects of xenobiotics on the molting process in crustaceans. Therefore, 20-HE has been recommended as a reference chemical when testing chemicals suspected to be ecdysone agonists [22]. Homobrassinolide, a steroid produced by several plants and some invertebrates to protect them against predators [102;103] was proposed as positive control for ecdysone antagonists [22]. To the best of my knowledge, no studies with homobrassinolide in relation to effects of potential EDCs on crustaceans have been published so far.

Baldwin et al. [104] exposed *D. magna* in a life-cycle test lasting 21 days to two ecdysteroids, 20-HE and PoA. The latter is an ecdysteroid found in crustaceans and

many plants and has a tenfold higher affinity to the ecdysteroid receptor than 20-HE [86]. PoA was almost ten times more toxic to *Daphnia magna* than 20-HE, indicating that the toxicity was in fact related to the ecdysteroid receptor. Daphnids were monitored for alterations in molting, fecundity and survival time. While both compounds did not have appreciable reproductive effects on daphnids, the study revealed some useful indicators of ecdysteroid exposure, e.g. incomplete ecdysis (inability to complete exuviations of the molt) leading to premature death. Though this effect only occurred at relatively high concentrations, Baldwin et al. suggested that incomplete ecdysis could be used in chronic toxicity testing to indicate that a particular chemical or effluent affect ecdysteroid function. PoA and other steroids produced by plants are possibly constituents of pulp and paper mill effluents and therefore may represent an environmental threat to arthropods living in waters near such industries.

20-HE was included as positive control for effects on molting in studies with several copepods species, e.g. *Tisbe battagliai* [105], *Tigriopus japanicus* [106], and *A. tonsa* **[3]** as well as in studies with *D. magna* [104;107-109]. While 20-HE significantly delayed molting in *D. magna* and also was toxic towards some copepods, no specific effects on naupliar development were seen in any of the three copepod species. NOECs for effects on naupliar development were 86.5 µg/l (*T. battagliai*), 10 µg/l (*T. japanicus*) and 1,500 µg/l (*A. tonsa*).

Since rapid degradation of 20-HE in the test medium could be one reason for not observing any effects on *A. tonsa* larval development **[3]** the experiment was repeated renewing the test solutions daily. The tested concentration range was 0.075-1.5 mg/l. A weak but significant stimulation of larval development was observed at 0.9 and 1.5 mg/l. This was the first observation of a stimulatory effect on larval development in my work with *A. tonsa* (**this study**, unpublished data). Taking into account that 20-HE is a naturally occurring hormone the determined effective concentrations seem to be rather high.

The fact, that 20-HE had no effect on larval development of *T. battagliai* and *T. japonicus* [105;106] together with the observation that it stimulated larval development in *A. tonsa* only at rather high concentrations suggests 20-HE not being the active molting hormone in these copepod species. Moreover, in *A. tonsa* PoA was 100 times more potent in stimulating LDR than 20-HE (see below), although its *in vitro* ecdysteroid activity is only ten times higher than that of 20-HE [86].

In the present Ph.D. work, PoA was studied in two experiments with *A. tonsa*. In the first test, a range from 2 to 500 µg/l was investigated. At 2 µg/l a significant stimulation of the LDR (32 %) was observed, whereas higher concentrations seemed to be toxic, no larvae reached a copepodite stage at concentrations ≥ 55 µg/l. Thus, a lower concentration range (0.1-10 µg/l) was tested in the second experiment. A stimulation occurred at all concentrations, but was only significant at 0.64 µg/l (30 %) and at 1.6 µg/l (41%) (**this study**, unpublished data). It would be interesting to utilise this stimulatory effect of a potent ecdysone receptor agonist in future mechanistic studies of suspected disrupters of the ecdysteroid system by a co-exposure with PoA.

Since ecdysteroids act as major endocrine-signalling molecules in crustaceans and are not only involved in molting [28] it may be expected that a chemical with (anti)ecdysteroid activity also will affect other hormonally regulated processes in crustaceans [94]. Support for this hypothesis was recently provided by Mu and LeBlanc [109], who demonstrated that the fungicide fenarimol not only altered intermolt duration but also embryo development in daphnids by interfering with ecdysteroid metabolism. Developmental abnormalities were associated with suppressed ecdysone levels in the embryos and could be prevented by co-exposure to 20-HE. Anti-ecdysteroid activity may provide a mean by which environmental chemicals impact crustacean species while not necessarily affecting vertebrates. However, in another study of Mu and LeBlanc [108], the vertebrate steroid hormone testosterone was shown to act as anti-ecdysteroid *in vitro* and *in vivo* giving rise to developmental toxicity similar to that observed for fenarimol. Interestingly, fenarimol is also known to act as androgen (via aromatase inhibition) as well as anti-androgen in vertebrate *in vitro* tests [110]. In a subsequent study, Mu and LeBlanc demonstrated synergistic effects of fenarimol on the embryotoxicity of testosterone [111]. Besides its embryotoxicity, testosterone delayed molting of daphnids indicated by a prolonged first intermolt period at 72 µg/l. This effect was mitigated by co-exposure to 20-HE. In *A. tonsa*, however, inhibition of larval development by testosterone occurred only at concentrations of 0.74 mg/l (EC_{10}) and 1.5 mg/l (EC_{50}), which were close to acute toxic levels for adults. Thus, there is no indication of any specific toxic effect of testosterone in *A. tonsa* **[3].**

4.2.2 Ecdysteroid-Mimics

The ecdysone-mimic RH 5849 (a bisacylhydrazine) is an insect growth regulator and acts as an ecdysteroid despite its nonsteroidal structure both *in vitro* [86;112] and *in vivo* [113] in insects. Evidence for a similar action in crustacean was provided by Clare et al. [90], who exposed larvae of the mud crab, *Rhithropanopeus harrisii,* and cypris larvae of the barnacle, *B. amphitrite,* to RH 5849 at concentrations ranging from 0.1-10 mg/l. RH 5849 accelerated molting in the crab larvae (0.1 and 1.0 mg/l) and

caused death due to incomplete ecdysis (10 mg/l). Attachment and metamorphosis of barnacle cyprids were enhanced at 10 mg/l of RH 5849. Physiological ageing of larvae induced by RH 5849 was supposed to be the reason for increased attachment. The observed effect on metamorphosis was consistent with the known stimulatory effect of 20-HE on this process in barnacles [114]. Tebufenozide is another bisacylhydrazine acting as an ecdysteroid receptor agonist, which is selectively toxic to lepidopteran (butterfly) species [115]. A few reports on the effects of this compound in crustaceans have been published. Song et al. [116] observed molt related acute toxicity at concentrations of 0.83 mg/l both in *D. magna* and *Artemia spp*., whereas Hirano et al. [117] found a NOEC of 10 mg/l in *D. magna* and *Americamysis bahia*. It would be interesting to test the effect of tebufenozide on larval development of *A. tonsa*.

4.2.3 Juvenile Hormone-Analogues

Another class of insect growth regulators are the juvenile hormone-analogues, which act as endogenous juvenile hormone and disrupt insect larval development and metamorphosis. Some of the JH-analogues, e.g. methoprene, have a structure similar to that of naturally occurring juvenile hormones from insects and to the crustacean hormone methylfarnesoate (MF). The insect hormone JH-III was a strong inhibitor of LDR in *A. tonsa* (EC_{50} = 100 µg/l, ACR>10) **[3]**. This observation is consistent with reports on the effect of JH-III on lobster larvae, in which a delay of metamorphosis occurred between 30 and 300 µg/l [118]. Two JH-analogues, fenoxycarb and methoprene, were tested in the present study. Fenoxycarb was even more potent in inhibiting larval development of *A. tonsa* than JH-III (EC10 =3 µg/l; EC_{50} = 27 µg/l) with a high ACR of about 30 (**this study**, unpublished data). In juvenile *R. harrisii*, fenoxycarb inhibited larval development (LOEC=48 µg/l) [119] and growth (100 µg/l) and reduced the total lipid content of the animals (50 µg/l) [120]. Thus, *A. tonsa* is more sensitive to fenoxycarb than *R. harrisii*. Neither fenoxycarb (**this study**, unpublished data) nor methoprene [86] showed *in vitro* ecdysteroid (anta)gonistic activity in the B_{II} test. Methoprene altered LDR in *A. tonsa* with an EC_{50} of 230 µg/l (ACR=6) (**this study**, unpublished data). These findings are in-line with reported results on the effect of methoprene on various other species, which revealed delayed larval development. For instance, development of *R. harrisii* [121] and the grass shrimp, *Palaemonetes pugio* [122] was affected at a concentration of 100 mg/l. A study with blue crabs, *Callinectes sapidus*, revealed that methoprene alters exocytosis and deposition of cuticular components in connection with delayed molt to the first crab [123]. Moreover, mysid shrimps, *Mysidopsis bahia*, exposed to methoprene had a reduced weight (62 µg/l), the release of the first brood was delayed and reproductive endpoints like number of offspring per brood were decreased [124]. In a study by Verslycke et al. [125], *N. integer* responded very sensitive to fenoxycarb (96-h LC_{50} = 0.53 mg/l) and methoprene

(96-h LC_{50} = 0.32 mg/l) exposure. These values are slightly below the 48-h LC_{50} found for *A. tonsa* (0.73 mg/l for fenoxycarb and 1.4 mg/l for methoprene) in this study. In addition, the short-term sublethal effect of methoprene on the energy and steroid metabolism of *N. integer* was evaluated [125]. Energy consumption was significantly induced at 100 µg/l, resulting in a lower cellular energy allocation in these animals. Methoprene exposure resulted in a concentration-dependent metabolic androgenisation. Verslycke et al. concluded that energy and testosterone metabolism of mysids are endpoints allowing to detect endocrine disrupting activity of chemicals after short-term exposure at environmentally relevant concentrations. Olmstead and LeBlanc [74] suggested that methoprene interferes with endocrine regulated processes in crustaceans by different mechanisms involving agonism and antagonism of various juvenoid receptor configurations. They demonstrated in this study with *D. magna* that methoprene has multiple mechanisms of toxicity leading to reduction in molt frequency, delayed first brood and reduced fecundity at concentrations in the low ng/l range.

In another study, Olmsteadt and LeBlanc [126] found that the crustacean hormone MF is a male sex determinant in *D. magna*, since continuous exposure to MF stimulated a concentration dependent production of male-broods. After having demonstrated that, they showed that JH-analogues, e.g. pyriproxyfen, mimic the action of MF resulting in production of male offspring [127].

4.2.4 Chitin Synthesis Inhibitors

A third group of insect growth regulators interfering with the molt process are the chitin synthesis inhibitors, which hamper normal development of the exoskeleton. These compounds do not act via the hormone system of the target organism indeed, but nevertheless they are interesting substances for comparison purposes because they often induce identical effects as substances affecting the ecdysteroid system. Diflubenzuron (Dimilin®) significantly inhibited molting in juvenile *U. pugilator*, at 20 and 200 µg/l [128]. A very interesting observation was made by Tester et al. [129], who exposed *A. tonsa* to Diflubenzuron. Females exposed to 1 and 10 µg/l produced fully developed eggs with moving nauplii visible inside the egg membrane. However, hatching of viable nauplii was significantly inhibited. Nauplii, which did hatch were abnormally shaped and failed to molt to the second naupliar stage. The nauplii were balloon-shaped with swollen appendages. Such balloon-shaped nauplii were never observed in *A. tonsa* at our laboratory, but were seen in *N. spinipes* exposed to 20-HE (Magnus Breitholtz, personal communication).

4.3 Vertebrate Steroid Hormones and Xeno-Hormones

It was already known from work published in the seventies and eighties that for example cadmium and various organochlorines such as lindane, DDT, PCBs, and PCDFs may interfere with molting in higher crustaceans, e.g. decapods [130-133]. But starting point for the present study were the fundamental publications of Baldwin and LeBlanc [134] as well as Zou und Fingerman [73;135], from which it was obvious that estrogens affect the molting process in *D. magna*, possibly by interacting with the ecdysteroid receptor. Results of the present thesis work confirmed these findings in principle, but clearly demonstrated, that larval development in *A. tonsa* is more sensitive than molting in *D. magna*.

LeBlanc and McLachlan [136] hypothesized that chemicals capable of binding to the vertebrate androgen receptor would also elicit toxicity to crustaceans by binding to specific steroid hormone receptors in an antagonistic manner. This hypothesis was tested by evaluating the effects of the antiandrogen cyproterone acetate on growth, molting, sexual differentiation, and reproduction of *D. magna*. Exposure of daphnids to concentrations of cyproterone acetate (130-2,100 µg/l) appreciably below those that elicited mortality reduced growth but had no effect on adult molting. Only at the highest concentration of 2,100 µg/l, molt frequency of juveniles was significantly reduced. These concentrations of cyproterone acetate had no effect on various developmental and maturation parameters. Cyproterone acetate also reduced the number of offspring produced by parthenogenetically reproducing daphnids, but this effect appeared to be a consequence of the reduced size of the daphnids and their inability to accommodate a brood of more than approximately 10 eggs. These results indicate that the antiandrogen cyproterone acetate specifically targets a process critical to growth of daphnids that is independent of molting. LeBlanc and McLachlan concluded that additional studies are needed to provide further evidence whether this molt independent growth reduction is an endocrine related toxicity to crustaceans that is associated with environmental antiandrogens. In *A. tonsa*, cyproterone acetate caused an inhibition of larval development with an EC_{10} of 81 µg/l and an EC_{50} of 156 µg/l (ACR=10) (**this study**, unpublished data), which are much lower than the LOEC of 2,100 µg/l reported for *D. magna* [136].

In 1995, Baldwin and LeBlanc [134] published a study on the effect of the xeno-estrogen diethylstilbestrol (DES) on *D. magna.* Chronic exposure of daphnids to 500 µg/l of DES reduced molting frequency among first-generation juveniles and decreased fecundity of second-generation daphnids. Interestingly, molting frequency was not affected in juvenile second-generation daphnids. Increased DES

metabolism/elimination capabilities of prenatally exposed organisms were suggested as explanation. Additionally, steroid hormone metabolic capabilities of *D. magna* were investigated using testosterone as model steroid [134]. The rate of elimination of two major hydroxylated metabolites of testosterone was significantly reduced, and elimination of glucose conjugates of testosterone was significantly elevated when *D. magna* was exposed to 500 µg/l of DES. Competitive binding of DES to the ecdysteroid receptor was hypothesised as a possible explanation for the reduction in molting frequency. The LOEC of 500 µg/l of DES for reduced molting frequency in juvenile daphnids [134] was high compared to the corresponding EC_{10} of 12 µg/l (EC_{50}=30 µg/l) found in *A. tonsa* (**this study**, unpublished data).

Like Baldwin and LeBlanc, Zou and Fingerman [73] also studied the effect of DES on molting of *D. magna.* Neonates were exposed to DES (0.05, 0.10 and 0.20 mg/l) and to the estrogenic pesticide endosulfan (0.05, 0.10 and 0.15 mg/l). Molting frequency was measured by visually inspecting each animal every 12 h and record if molting had occurred. The completion of four molts took significantly longer in daphnids exposed to the two highest concentrations of both compounds. It should be noted that a high mortality of 20 to 50% occurred in the exposed groups compared to 20 % in the control. Next, Zou and Fingerman [135] investigated DEP, 4-OP, lindane, Aroclor 1242 and PCB29 at relatively high concentrations close to their acute toxic levels. DEP (LOEC=22.4 mg/l, ACR=3); Aroclor 1242 (LOEC=0.05 mg/l, ACR=5); and PCB29 (LOEC=0.20 mg/l, ACR=6) significantly inhibited molting. Lindane (NOEC=0.20 mg/l) and 4-OP (NOEC=0.04 mg/l) had no effect on molting. In another study with *D. magna* [137] following the method described by Zou and Fingerman [73] there was no effect of BPA on molting at concentrations up to 3.2 mg/l. In contrast, the EC_{50} values for inhibition of *A. tonsa* larval development were 0.50 mg/l of BPA and 0.013 mg/l of 4-OP **[3]**. DEP inhibited molting in *D. magna* with a LOEC of 22.4 mg/l [135], whereas 12 mg/l of DEP had no effect. Compared to these results, the EC_{50} for inhibition of *A. tonsa* larval development was just 10 mg/l **[3]**. Thus, the EC_{50} values for *A. tonsa* are lower than the reported NOECs found in *D. magna* for all three chemicals (BPA; 4-OP, and DEP). This demonstrates again that *A. tonsa* often responds much more sensitive to chemicals, which interfere with growth and molting processes than *D. magna*. The experiments with *D. magna* performed by Zou and Fingerman [73;135] and the larval development test with *A. tonsa* **[3;4;6;8]** have a lot in common. For instance, daphnids were exposed from neonates until the fourth instar (day 5-7 of the experiment). Thus, exposure was completed before reproduction started. Advantages of the *A. tonsa* test compared to the method applied by Zou and Fingerman are the low expenditure of human labour, its low cost and rapidness. Thus, high numbers of exposure concentrations and replicates are feasible, which allow the estimation of

concentration-response curves. From the *D. magna* experiments only NOECs and LOECs are obtained. Concentration-response relationships are beneficial because they strengthen the confidence with which the observed effects can be described.

In-line with Baldwin and LeBlanc [134] Zou and Fingerman supported the hypothesis that some compounds capable of disrupting hormonal processes in vertebrates can also interfere with endocrine-mediated molting processes in arthropods, presumably by acting as antagonists of endogenous ecdysteroids [135]. For example, E2 and DES were recently shown to inhibit the binding of ^{3}H-labelled PoA in an insect cell-line [138]. Alternative mechanisms of endocrine disruption such as the inhibition of ecdysteroidgenesis in the Y-organ were, however, not excluded by Zou and Fingerman [135].

Five of the compounds tested for their ability to delay molting in *D. magna* (4-OP, DEP, DES, lindane and BPA) were later also investigated for their ecdysteroid (anta)gonistic activity in the B_{II} cell assay [86]. Lindane and BPA acted as anti-ecdysteroids *in vitro*. Just these two compounds did not affect molting in *D. magna* [135;137]. This indicates that other mechanisms than interaction with the ecdysteroid receptor were responsible for the delay in molting induced by DES and DEP in *D. magna*. However, BPA inhibited larval development in *A. tonsa* at concentrations much below the NOECs reported for *D. magna* **[3]**, which in turn suggests different modes of action of BPA in *D. magna* and *A. tonsa*, respectively.

In 1999, Zou and Fingerman published three studies [72;139;140], which finally provided a more specific explanation for the previously hypothesised ability of xeno-estrogens to inhibit molting in *D. magna*. The subject of the first of these three papers [139] was the activity of a chitinolytic enzyme during the molting cycle in *U. pugilator*. Separation of the old exoskeleton, a process termed apolysis, occurs when the crustacean enters the proecdysis stage. Partial digestion of the old chitinous cuticle is brought about by the molting fluid in the apolytic space. This fluid is secreted by the epidermis and contains proteinases and two chitinolytic enzymes, chitinase and chitiobase. The authors investigated the activity of chitobiase in the epidermis and hepatopancreas during the molting cycle. The patterns of chitobiase activity correlated well with the hemolymph titer of ecdysteroids during the molting cycle suggesting that chitobiase activity in both tissues is regulated at least in part by the steroid molting hormones. Subsequently Zou and Fingerman studied the effects of exposure of *U. pugilator* to Aroclor 1242, DES, endosulfan, and 4-OP [140] as well as to 4-*tert*-OP, DEP, and PCB29 [72] on chitobiase activity. All these xeno-estrogens did inhibit

chitiobase activity in the epidermis and/or hepatopancreas significantly, but 4-OP only at high exposure levels. Because chitobiase is necessary for the partial digestion of the chitinous exoskeleton as part of the molting process, inhibition of this enzyme by estrogenic agents can account for at least some of the slowing down of molting that occurs when crustaceans are exposed to them. The authors concluded the following: Since chitinolytic enzymes are products of ecdysteroid regulated genes in arthropods, the decline in chitobiase along with the delayed molting strongly suggests that those xenobiotics disturb the Y-organ-ecdysteroid receptor axis. Such disturbance may be caused by an interaction between ecdysteroid receptors and steroid-mimics whereas the steroid-mimics act as antagonists of endogenous steroid molting hormones or arise from the interference of those compounds with the synthesis and excretion of ecdysteroids, or both.

Whether such interactions described for a decapod can also take place in smaller crustaceans remains to be studied. There are various studies, primarily with copepods, in which no dose-related effects on development were reported in reference to interaction of estrogens with the molting process. For example, Bechmann [81] studied effects of nonylphenol (NP) in a life-cycle test with *T. battagliai.* The tested concentrations ranged from 31 to 500 µg/l. The highest concentrations (125 and 500 µg/l) were lethal to nauplii, but no effects on development, sex ratio and fecundity were observed in this study. Moreover, Hutchinson et al. studied effects of DES [82] as well as E1, E2 and EE2 [105] on the life-cycle of *T. battagliai*. The turn from nauplii to copepodites was used to examine the effect of DES on development; a NOEC of 100 µg/l of DES was determined. However, DES affected reproductive parameters and survival (21-d LOECs=100 µg/l). E1, E2, and EE2 were tested up to concentrations of 100 µg/l, no effects on development, sex-ratio and fecundity of *T. battagliai* were observed. Breitholtz and Bengtsson [141] examined whether E2, EE2 and DES affect mortality, larval development rate, fecundity and sex ratio of *N. spinipes* in a life-cycle test similar to the method applied in the present study **[5;7]**. The only observed effect was a reduced percentage of gravid females at 30 µg/l of DES, which was the highest concentration tested. Neither E2 (NOEC=160 µg/l) nor EE2 (NOEC=50 µg/l) induced any measurable effects on larval development rate, fecundity and sex ratio. The same estrogens studied by Hutchinson et al. [82;105] as well as Breitholtz and Bengtsson [141] were investigated in the larval development test with *A. tonsa*, again demonstrating that this copepod is more sensitive than other crustaceans **[3]**. Significant effects of estrogens on development of *A. tonsa* were seen at concentrations generally below or close to the NOECs obtained for inhibition of larval development of *N. spinipes* and development of *T. battagliai.* For example, LC_{10} values were 46 µg/l of EE2, 37 µg/l of E2 **[3]** and 12 µg/l of DES (**this study**, unpublished data). However, only for EE2 and

DES the calculated ACR (LC_{50}/EC_{50}) were above ten. Interestingly, EE2 has been shown to be a weak ecdysteroid antagonist *in vitro* [86].

The usefulness of small crustaceans like copepods for the evaluation of EDCs with regard to invertebrates has increasingly being recognised during the last years and has been reflected in recent publication on that issue, e.g. [5;7;78;81;82;95;105]). Partial and full life-cycle tests with copepods were established using e.g. the marine copepod *A. tonsa* **[4;6]**, the intertidal copepod *T. japonicus* [106] the estuarine copepod *Eurytemora affinis* [142], the freshwater copepod *Bryocamptus zschokkei* [143] and the brackish copepod *N. spinipes* **[5;7]**.

For example, Marcial et al. [106] studied effects on development and reproduction in two successive generations of *T. japonicus* induced by continuous exposure to environmentally relevant concentrations of E2, BPA, 4-NP, 4-*t*-OP and 20-HE. Less than 24-h-old nauplii were exposed to four sublethal concentrations of those compounds for 21 d. Fecundity, sex ratio, and survival were not significantly affected; the NOEC of all tested compounds was 10 µg/l. However, all compounds affected development (time to reach copepodite stage and sexual maturity) in the first generation. The LOECs for effects on larval development were 1 µg/l of E2, 1 µg/l of 4-NP, 0.1 µg/l of BPA, 0.1 µg/l of 4-*t*-OP, and 10 µg/L of 20-HE. Effects were apparent in the second generation, too. These values are low compared to other studies with copepods. For example, a LC_{10} of 5 µg/l of OP was obtained in the larval development test with *A. tonsa* **[3].** It is, however, well known that branched alkylphenols have a higher *in vitro* estrogenic potency than the respective linear ones. NP affected timing of larval development of the barnacle *Elminius modestus* at very low NP concentrations ranging from 0.01-10 µg/l [144]. The LOECs for a delay of larval development obtained in the study with *T. japonicus* [106] seem extremely low compared to the acute toxic levels. ACRs calculated from 48-h LC_{50} and LOECs were as high as 500 (4-NP), 3,000 (E2), 6,000 (4-*t*-OP) and 40,000 (BPA). Both, BPA and NP act as anti-ecdysteroid *in vitro*, whereby the activity of BPA is higher than that of NP [86]. This might partly explain why the ACR of BPA was much higher than those of the other estrogens. However, since the estrogens were investigated only at four different concentrations, no concentration response relationships were established.

Five EDCs were investigated in a transgenerational life-cycle test with the estuarine copepod *E. affinis* [142] considering the endpoints survival, naupliar development, sex ratio, fecundity and number of broods. The most sensitive endpoint was larval development. Naupliar development was delayed (LOEC) at 109 µg/l of DEHP, 25 µg/l

of atrazine, 12 µg/l of benzo[a]pyrene, 7 µg/l of NP, and 6 µg/l of E2. Unfortunately, only a very few concentrations were tested so that no quantitative concentration response curves could be established allowing the calculation of EC_X values.

A full life-cycle test was performed to measure the effect of lindane (3.2-3,200 µg/l) on survival, development and reproduction of the freshwater copepod *B. zschokkei* [143]. Equi-proportional development, which assumes that each molt stage represents a certain proportion of the total development time and is not affected by processes that influence metabolism such as temperature and food quality, was used to determine the mode of action of lindane on developmental parameters. The time to reach the adult stage was significantly longer at 100 µg/l of lindane compared to the controls, but remained equi-proportional regardless of lindane exposure. Prolonged development times, therefore, were not due to a direct effect of lindane on the molting process but were probably caused by reduced food intake or increased metabolism elicited by the stress imposed by toxicant exposure. Incorporating a copepod growth model (equiproportional development) into the life-cycle test design provided useful information on the dominant mode of action of lindane [143].

4.4 Model Compounds

After having demonstrated that larval development of *A. tonsa* was a very sensitive endpoint for evaluating effects of chemicals that might interfere with the endocrine system of crustaceans, the larval development test has been applied to two groups of emerging environmental contaminants, brominated flame retardants (BFRs) and synthetic musks, known or suspected to be potential endocrine disrupters. In addition, these substances were studied in the full life-cycle test with *N. spinipes*. To test for ecdysteroid agonistic/antagonistic activity the bioassay with the ecdysteroid-responsive *D. melanogaster* B_{II} cell line was applied. The results of these investigations have been presented and discussed in detail in the papers **[4-7]**. Therefore, just a summary of the most important results will be given below

Brominated Flame Retardants (BFRs) have entered the aquatic environment and have now been detected in freshwater and marine organisms as well as sediments from many locations around the world [24]. Toxicity studies with BFRs have mainly been performed on mammals as yet. Their acute toxicity seems to be low, but there is concern about various chronic effects. Some BFRs hav shown to act as reproductive, developmental, immuno- and neurotoxins. They induce certain liver enzymes and act as endocrine disrupters, especially on the thyroid system, but estrogen receptor binding has

also been reported [24;145-147]. For the time being, knowledge about the impact of BFRs on aquatic organisms is very limited. A primary contribution of the present work with BFRs was to establish data on their ecotoxicity.

Subchronic effects of tetrabromobisphenol A (TBBPA), tribromophenol (TBP) and four polybrominated diphenyl ethers, PBDEs (BDE-28, BDE-47, BDE-99 and BDE-100) on larval development of *A. tonsa* were studied in paper **[6]**. For TBBPA and TBP 5d-EC_{50} values for inhibition of the larval development rate were 125 and 810 µg/l, respectively, whereas the PBDEs were much more potent with 5d-EC_{50} in the low µg/l range (1.2 µg/l for BDE-100; 4.2 µg/l for BDE-99; 13 µg/l for BDE-28 and 13 µg/l for BDE-47). These concentrations were up to two orders of magnitude below the 48h-LC50 for acute adult toxicity (108 µg/l for BDE-28; 400 µg/l for TBBPA; 520 µg/l for BDE-100; 705 µg/l for BDE-99; 1,500 µg/l for TBP and 2,370 µg/l for BDE-47). To distinguish between general toxicological and endocrine-mediated toxic effects, the BFRs were assessed *in vitro* for ecdysteroid agonistic/antagonistic activity. The pentabrominated diphenyl ethers BDE-99 and BDE-100 showed weak ecdysteroid antagonistic activity. Thus, these PBDEs may be regarded as potential endocrine disrupters in invertebrates. The combination of *in vitro* assays and subchronic biotests with ecologically important crustacean species was shown to be a rapid and cost-effective tool when screening for sublethal effects of chemicals.

The full life-cycle test with *N. spinipes* was used to study effects of BDE-47, -99 and -100 on LDR and population growth rate **[7]**. LDR significantly decreased in copepods exposed for 6 days to nominal concentrations ≥ 13 µg/l of BDE-47 and ≥ 30 µg/l of BDE-99. High ACRs (up to 340) between adult acute and juvenile subchronic toxicity were observed. Exposure over the full life cycle showed in general that population growth rate was a less sensitive endpoint than LDR. Still, the population growth rate in copepods exposed to 40 µg/l of BDE-47 was significantly reduced compared to the controls. Partitioning experiments with ^{14}C-labelled BDE-47 and BDE-99 in the test system showed that the major fractions of both compounds (50-80%) were adsorbed to particulate material. These findings indicate that development and reproduction in *N. spinipes* are sensitive to the tested PBDEs and that ingestion of particle-adsorbed PBDEs most likely is the predominant route of exposure in this species.

A nitro musk (musk ketone - MK) and three polycyclic musks (Tonalide - AHTN, Galaxolide - HHCB and Celestolide - ADBI) were tested for acute and subchronic effects on *A. tonsa* **[4]** using the methods described elsewhere **[3;6]**. The inhibition of LDR after 5 days exposure was demonstrated to be a very sensitive endpoint, with

5d-EC_{50} values as low as 26 µg/l (AHTN) 59 µg/l (HHCB), 66 µg/l (MK) and 160 µg/l (ADBI), respectively. These values were generally more than one order of magnitude below the 48-h LC_{50} values found for adults, which were 0.47 mg/l (HHCB), 0.71 mg/l (ADBI), 1.32 mg/l (MK) and 2.5 mg/l (AHTN). Since the synthetic musks strongly inhibited larval development in *A. tonsa* at low nominal concentrations, they should be considered as very toxic. The larval development test with *A. tonsa* was shown to be able to provide important aquatic toxicity data for the evaluation of the environmental risk of synthetic musks, for which there has been little ecotoxicological information available regarding crustaceans so far. It was suggested that subchronic and chronic copepod toxicity tests due to their high sensitivity to potential EDCs and other specific toxic compounds should be incorporated in the risk assessment of environmental pollutants routinely.

The same four synthetic musks were investigated in the full life-cycle test with *N. spinipes* **[5]**. The LDR, was recorded after 7-8 days exposure of juveniles and was significantly decreased in copepods exposed to sublethal concentrations of MK, ADBI and HHCB. However, none of the AHTN concentrations had any effect on larval development. The lowest HHCB concentration (0.02 mg/l), which affected juvenile development, was about 100 times below the adult 96h-LC_{50} value of 1.9 mg/l. In the B_{II} cell assay, none of the four musks showed any agonistic or antagonistic activity. This indicates that the decrease in larval development rate was due to general toxic effects rather than steroid receptor-mediated endocrine disruption. A modified Euler-Lotka equation was used to calculate a population-level endpoint, the intrinsic rate of natural increase, from individual life-table endpoints, i.e. mortality rate, time of release of first brood, sex ratio, the fraction of ovigerous females among all females as well as the number of nauplii per ovigerous female. This population-level endpoint was affected significantly at the second highest MK concentration (0.1 mg/l) only. At the highest MK (0.3 mg/l) and ADBI (0.3 mg/l) concentrations, all copepods were dead at the end of the exposures. The results demonstrated that it is possible to obtain population-level data from the full life-cycle test with *N. spinipes*.

Table 4 Literature survey on endocrine disrupting effects in crustaceans comprising subclass and species, name of the chemical studied or site, effects observed including effective concentrations, as well as reference.

Species (life stage)	Compound/site	Effect, concentration (range)	Reference	Remark
Amphipods				
Corophium volutator (juvenile-adult)	4-NP	Reduced growth, increased female fertility and antennae length, > 10 µg/l	[148]	
Echinogammarus marinus	Polluted sites, East Scotland	Intersexuality, demasculinisation	[149]	Field study
Gammarus fossarum	Sampling sites near and away sewage treatment work	Intersexuality	[150]	Field study
Gammarus pulex (adult)	Cadmium	Precopulatory behaviour (reduced time to separation of precopulatory pairs), 5-45 µg/l	[151]	
Gammarus pulex (adult)	E2, BPA	Precopulatory behaviour, NOEC=3.7 mg/l E2, LOEC = 8.4 mg/l BPA	[152]	
Gammarus pulex (juvenile)	Lindane	Reduced growth (weight), 6.1 µg/l	[153]	
Gammarus pulex (adult)	Lindane, DCA, polluted sites near Cardiff, Wales	Precopulatory behaviour (reduced time to separation of precopulatory pairs), 50 and 100 µg/l lindane, 3 and 6 mg/l DCA	[154]	Field study
Gammarus pulex (adult)	Downstream sewage treatment work	Abnormal oocyte structure during vitellogenesis, reduced body size and reduced male/female size differences	[155]	Field study
Hyalella azteca (multi-generational)	EE2	Inhibition of male secondary sexual characteristics in F1 generation, 0.1 and 0.32 µg/l; histological aberrations of reproductive tract in post F1-generation males, 0.1 – 10 µg/l	[156]	
Hyalella azteca (adult)	Lindane	Reduced growth and population size, reduced number of precopulatory pairs and gravid females, 13.5 µg/l	[157]	
Hyalella azteca (adult)	Lindane	Direct and indirect separation of precopulatory pairs, LOEC = 24 and 17 µg/l, respectively	[158]	

Table 4 continued

Leptocheirus plumulosus (juvenile-adult)	Sewage impacted sediment, Jamaica Bay	Decrease in reproduction (by 50%)	[159]	Field samples
Monoporeia affinis	Contaminated sediment (heavy metals, PCBs, PAHs)	Delayed or interrupted sexual maturation	[160]	Field samples
Cladocerans				
Cerodaphnia dubia (adult)	Styrene dimers and trimers (a), α- ecdysone (b), β-ecdysone (c), cyasteron (d), PoA (e), E1 (f), testosterone (g), NP (h), BPA (i)	Decrease in reproduction by 25 %, 0.04-1.7 µg/l (a), 0.10 µg/l (b), 0.36 µg/l (c), 0.26 µg/l (d), 1.05 µg/l (e), > 10,000 µg/l, (f), 2,800 µg/l (g), 280 µg/l (h), 4000 µg/l (i)	[161]	
Chydorus piger (life-cycle)	Cadmium (sediment bound)	Delay in first reproduction, population growth rate reduced	[162]	
Daphnia carnita (life-cycle)	Pyriproxyfen	Suppressed growth, reduced reproduction (by 80%), 0.01 mg/l	[163]	
Daphnia galeata	Dieldrin, endosulfan	Reduced male production, 50-600 µg/l dieldrin (no effect of endosulfan)	[164]	
Daphnia galeata (multi-genertional)	4-NP	Population level effects, 50% reduction of the intrinsic rate of natural increase in F0 generation, 65 µg/l, and F1 generation, 82 µg/l,	[165]	
Daphnia magna	Pentachlorophenol	Reduced biotransformation/elimination of testosterone, reduced fecundity, 0.12 –0.5 mg/l	[166;167]	
Daphnia magna	Methoxychlor (a), NP (b), DDT (c), TBT (d), E2 (e)	Reduced of molting frequency, 1 µg/l (a); no effect on molting frequency 25 µg/l (b), 1 µg/l (c and d), 270 mg/l (e); effects on sex-determination observed depending on food concentration	[168]	
Daphnia magna (life-cycle)	20-HE, PoA	Incomplete ecdysis, no effect on molting frequency or reproduction, 31-125 µg/l 20-HE; incomplete ecdysis, reduced fecundity in F1 generation, 1.6-13 µg/l PoA	[104]	

Table 4 continued

Daphnia magna (adult)	NPEO	Metabolic androgenisation (by altering metabolic elimination of testosterone), 5 mg/l	[169]
Daphnia magna (adult)	4-*n*-NP	Metabolic androgenisation (disrupted testosterone metabolism), 25 and 100 µg/l; reduced fecundity, 100 µg/l	[69]
Daphnia magna (adults)	20-HE	Modulation of testosterone (exogenous) biotransformation by detoxication enzymes, 2 mg/l	[65]
Daphnia magna (multi-genertional)	DES	Reduced molting frequency in F0 generation, decreased fecundity in F1-generation, altered steroid hormone metabolic capacities in F1-generation, 0.5 mg/l	[170]
Daphnia magna (neonate-adult)	BPA	No effect on molting cycle and fecundity, 0.3 and 3 µg/l	[137]
Daphnia magna (juvenile)	Lindane	Altered carbohydrate metabolism, 0.056 - 0.75 mg/l (less sensitive than reproductive and population level endpoints)	[171]
Daphnia magna	4-NP	Altered secondary sexual characteristics, 100 µg/l	[172]
Daphnia magna (neonate-adult)	DEHP	Reduced fecundity, 800 µg/l; changes in biochemical composition, 158 and 800 µg/l	[70]
Daphnia magna adult-neonate	*o,p'*-DDT (a), di-*n*-buthyl phtalate (b), toxaphene (c) *p,p'*-DDE (d), linuron (e), diflubenzuron (f)	Increased male production, 50 and 100 µg/l (a); decrease in clutch size, 50 µg/l, no effect on growth (a), no effect on sex ratio, growth and fecundity at 0.001 to 100 µg/l (remaining compounds)	[75]
Daphnia magna (neonate-adult)	Cyproterone acetate	Reduced growth (independent of molt), 0.5-2 mg/l; reduced molting frequency in juveniles, 2 mg/l; reduced brood size (due to reduced body size), 1 and 2 mg/l	[136]
Daphnia magna (neonate-adult-embryo)	4-NP (a), testosterone (b)	Embryo developmental abnormalities 100 and 200 µg/l (a), 1.2-5.8 mg/l (b); increased fecundity 100 and 200 µg/l (a),	[173]
Daphnia magna (embryogenesis)	Fenarimol (a), testosterone (b)	Abnormal embryonic development, 1.3 mg/l (a), 3.5 mg/l (b), synergistic effect	[111]

Table 4 continued

Daphnia magna (neonate-adult-embryo)	Fenarimol	Lowered ecdysone levels after embryonic or neonatal exposure, delay in molting, embryo developmental abnormalities (effects are mitigated by co-exposure to 20 HE), reduced fecundity, 0.03-1 mg/l	[109]	
Daphnia magna (neonate-juvenile; adult-embryo)	Testosterone (a), E1 (b), androstenedione (c)	Reduced fecundity2.3 mg/l (a), decreased molting frequency and embryo developmental toxicity (both effects mitigated by co-exposure to 20-HE), 7.2 mg/l (a), no effect on fecundity 2.2 mg/l (b) and (c), 2.3 mg/l	[108]	
Daphnia magna	MF, pyriproxifen	Stimulated production of male offspring, 0.8-800 nM MF, 0.30 nM pyriproxifen	[127]	
Daphnia magna	DES (a); methoprene (b), androstenedione (c)	Stimulation of female secondary sex characteristics 800 µg/l (a), 24 µg/l (b), ≥1.7 mg/l (c)	[174]	
Daphnia magna (neonate-adult)	Methoprene	Reduced growth rate and fecundity, Delay in sexual maturation, 1.6-16 µg/l; reduced molting frequency, 0.06 µg/l	[74]	
Daphnia magna (neonate-adult)	Methoprene	Changes in sexual reproductive cycling (continuance of pathogenesis), inhibition of resting egg production, stimulation of male production, 62-50 µg/l	[175]	
Daphnia magna	Pyriproxifen (a), fenoxycarb (b), MF (c), JH-III (d), methoprene (e)	Reduced reproduction, sex ratio dominated by males, 0.33 µg/l (a and b), 3.7 µgI/l (c), 330 µg/l (d), 1,300 µg/l (e)	[176]	
Daphnia magna	TCDD	Reduced fecundity, 0.1-1,0 ng/l	[177]	
Daphnia magna (life-cycle)	4-NP	Arrested egg development, 800 and 1000 µg/l; late stage neonate deformities, 50 and 200 µg/l; decrease in fecundity (no effect on juvenile molting frequency) 12-50 µg/l	[71]	
Daphnia magna (juvenile)	Endosulfan, DES	Molt inihibition, 1 and 1.5 mg/l endosulfan, 0.1 and 0.2 mg/l DES	[73]	
Daphnia magna	PCB29 (a), Aroclor 1242 (b), DEP (c), 4-OP (d), lindane(e)	Prolonged time period to complete four molts, 0.2 mg/l (a), 0.05 and 0.10 mg/l (b), 22.4 mg/l (c), NOEC = 0.04 mg/l (d), NOEC = 0.20 mg/l (e)	[135]	
Daphnia sp.	Lake Mendota, Wisconsin, U.S.	Decline in maximum frequency of males since 1940	[93]	Field study

Table 4 continued

Daphnia pulex	Methoprene (a), 20-HE (b)	Decrease in all-male broods, increase in all-female broods 10 and 100 µg/l (a) and 100 µg/l (b); decrease in all-male broods, decrease in all-female broods, 1 and 10 µg/l (b)	[178]
Daphnia pulex,	Diuron	Decreased reproduction, LOEC = 7.7 mg/l	[179]
Daphnia pulicaria (adult)	Atrazine	Increased male production, 0.5 and 10 µg/l	[180]
Moina macrocopa (neonate-adult)	(S)-methoprene	Reduced fecundity, 0.05-0.5 mg/l	[181]
Copepods			
Acartia tonsa (egg-juvenile)	TBT	Inhibition of larval development, EC10 =1 ng/l, EC50=3 ng/l	[78]
Acartia tonsa	Diflubenzuron	Reduced egg viability, morphological abnormalities, stage I nauplii unable to molt, 1-10 µg/l; reduced fecundity,100 µg/l	[129]
Acartia tonsa (egg-juvenile)	E2 (a), E1 (b), testosterone (c), progesterone (d), 20-HE (e), JH-III (f), flutamide (g), tamoxifen (h), hydroxyflutamide (i), EE2 (j), 4-OP (k), BPA (l), NPEO (m), DEHP (n) 3,4-DCA (o)	Inhibition of larval development, EC10 (mg/l): 0.37 (a), 0.25 (b), 0.74 (c), 0.017 (f), 0.135 (g), 0.0087 (h), 0.0053 (i), 0.046 (j), 0.0052 (k), 0.10 (l), 0.022 (m), 5.0 (n), 0.38 (o); EC50 (mg/l): 0.72 (a), 0.41 (b), 1.5 (c), 0.102 (f), 0.48 (g), 0.049 (h), 1.4 (i), 0.088 (j), 0.013 (k), 0.55 (l), 0.15 (m), 10 (n), 0.54 (o); NOEC (mg/l): 1.0 (d), 1.5 (e); acute-to-chronic ratio >10 (f, g, j, k, m, o)	**[3]**
Acartia tonsa (egg-juvenile)	PoA (a), methoprene (b), fenoxycarb (c), *p,p'*-DDE (d), CPA (e), vinclozolin (f) DES (g), ICI 182 (h), NP acetate (i)	Stimulation of larval development at 0.0006-0.0016 mg/l (a); inhibition of larval development rate EC10 (mg/l): 0.166 (b), 0.003(c), 0.0001 (d), 0.08 (e), 0.63 (f), 0.02 (g), 1.65 (h), 0.7 (i); inhibition of larval development EC50 (mg/l): 0.226 (b), 0.027 (c), 0.002 (d), 0.156 (e), 3.53 (f), 0.030 (g), 1.98 (h), 3.60 (i)	[**this study,** unpublished data]
Acartia tonsa (egg-juvenile)	Synthetic musks: MK (a), HHCB (b), AHTN (c), ADBI (d)	Inhibition of larval development, EC10 (µg/l): 10 (a), 37 (b), 7 (c), 36 (d); EC50 (µg/l): 66 (a), 59 (b), 26 (c), 160 (d); acute-to-chronic ratio: 20 (a), 8 (b), 27 (c), 16 (d);	**[4]**

Table 4 continued

Acartia tonsa (adult)	20-HE (a), JH-III (b), E1 (c), E2 (d), EE2 (e), progesterone (f), DES (g), testosterone (h), methyl-testosterone (i), flutamide (j), NPEO10 (k), DCA (l), BPA (m), TBBPA (n)	Reduced female fecundity (measured as egg production) EC10 (mg/l): 0.1 (a), 0.003 (b), 0.27 (d), 0.042 (f), 0.03 (h), 0.46 (i), 0.265 (k), 0.734 (l), 0.021 (m), 0.112 (n) NOEC (mg/l): 0.1 (c), 0.1 (e), 0.1 (g), 1.0 (j) EC50 (mg/l): 0.65 (b), 0.98 (d)	[**this study,** unpublished data]	
Acartia tonsa (egg-adult)	E2, BPA	Accelerated sexual maturation of females, 23 µg/l E2; 20 mg/l BPA	[182]	
Acartia tonsa (egg-juvenile)	PBDEs: BDE-28 (a), BDE 47 (b), BDE-99 (c); BDE-100 (d); TBP (e) TBBPA (f)	Inhibition of larval development, EC10 (µg/l): 4.6 (a), 2.4 (b), 0.39 (c), 0.02 (d), 270 (e), 13 (f); EC50 (µg/l): 12.8 (a), 12.5 (b), 4.2 (c), 1.2 (d), 811 (e), 125 (f); acute-to-chronic ratio: 190 (b), 450 (d), 170 (c); weak ecdysteroid activity of (c) and (d) in vitro (B_{II} test)	**[6]**	
Amphiascus tenuiremis (trans-generational)	Fipronil	Reduced reproduction due to male infertility, 0.63 µg/l	[183]	
Amphiascus tenuiremis (juvenile)	Fipronil	Induction of lipovitellin in females (not in males), 0.6 µg/l	[57]	
Amphiascus tenuiremis (life-cycle)	Fipronil	Strong inhibition of reproduction, delayed development, ≥0.22 µg/l; decline in modelled population size, 0.16 mg/l	[184]	
Amphiascus tenuiremis	Atrazine	Reproductive failure in F1 generation, 25 and 250 µg/l; copepod malformation (F1 generation) and decline in modelled population size (2.5, 25 and 250 µg/l)	[185]	
Bryocamptus zschokkei (life-cycle)	Lindane	Delayed development (but still equiproportional), 100 µg/l; reduced egg production and offspring viability, 32 µg/l	[143]	
Harpacticoid copepods, four species	Firth of Fourth, Scotland	Intersex	[92;186]	Field study
Nitocra spinipes (neonate-adult)	E2 (a), EE2 (b), DES (c)	No effect on larval development, sex ratio and fecundity, 0.5-50 µg/l (a and b); reduced percentage of gravid females, 30 µg/l (c)	[141]	

Table 4 continued

Nitocra spinipes (life-cycle)	PBDEs: BDE-47 (a), BDE-99 (b), BDE-100 (c)	Inhibition of larval development, ≥13 µg/l (a), ≥ 30 µg/l (b), acute-to-chronic ratios above 300; population growth rate reduced, 40 µg/l (a)	**[7]**
Nitocra spinipes (life-cycle)	Synthetic musks: MK (a), HHCB (b), AHTN (c), ADBI (d)	Inhibition of larval development, , ≥30 µg/l (a), ≥ 20 µg/l (b), 100 µg/l (c), acute-to-chronic ratio 100 (b); population growth rate reduced, 100 µg/l (a); no ecdysteroid activity in vitro (a, b, c, d)	**[5]**
Tisbe battagliai (life-cycle)	20-HE, DES	Reduced reproduction (27 µg/l 20 HE); no breeding females ≥86.5 µg/l 20-HE, 100 µg/l DES, (no effect on naupliar development and sex ratio)	[82]
Tisbe battagliai (life-cycle)	E2, E1, EE2	No effect on development, sex ratio and reproduction, 0.1-100 µg/l for all compounds	[105]
Tisbe battagliai (multi-generational)	NP	No effect on demographic parameters, 31µg/l; population is collapsing, 62 µg/l	[81]
Tigriopus japonicus (multi-generational)	E2 (a); BPA (b), 4-NP (c), *p-t*-OP (d), 20-HE (e)	Delay in completion of naupliar stages (F1 generation more sensitive), 1 µg/l (a), 1 and 10 µg/l (c), 0.1-10 mg/l (b), 0.1 and 1 µg/l (d); no developmental effects of (e); no effects on fecundity and sex ratio, except 0.01 µg/l (a) (more females); tested range for all compounds 0.01 – 10 µg/l	[106]
Decapods			
Balanus amphitrite (cyprid larva)	E2, 4-NP	Inhibition of barnacle settlement, 0.01-10 µg/l, both compounds	[187]
Balanus amphitrite (cypris larva)	RH 5849	Enhanced attachment and metamorphosis, 10 mg/l	[90]
Balanus amphitrite (cypris larva)	E2; 4-*n*-NP	Induction of cypris major protein (vitellin-like protein), 1 µg/l, both compounds	[55]
Callinectes sapidus (larva-adult)	Methoprene	Reduced hatching success, lethargic behaviour, inhibition of metamorphosis, 0.6-3 mg/l	[123]
Cardina rajadhari (adult)	TBT oxide	Decreased calcium resorption from the exoskeleton, 15 – 40 ng/l	[188]

Table 4 continued

Chasmagnathus granulata (adult)	Cadmium, copper	Hypoglycemia (inhibition of CHH secretion), 0.5 mg/l Cd, 1 mg/l Cu	[189]	
Chasmagnathus granulata (juvenile)	Cadmium	Reduced molting frequency, 10 and 50 µg/l	[190]	
Eliminus modestus (larva)	Farnesol	Disturbed attachment and metamorphosis, 5 x 10^{-7} – 10^{-6} (v/v)	[191]	
Elminius modestus (neonate-cypris larva)	E2; 4-*n*-NP	Changes in DNA profiles of larval barnacles, 0.1-10 µg/l (both compounds)	[96]	
Elminus modestus (larva)	4-*n*-NP E2	Inhibition of larval development 0.01-10 µg/l 4-n-NP; Reduced growth, 10 µg/l E2	[144]	
Eurytemora affinis	E2 (a), Benzo[a]pyrene (b), 4-NP (c), DEHP (d), atrazine (e)	Inhibition of larval development, no influence on fecundity and number of brood, 6 µg/l (a), 12 µg/l (b), 7 µg/l (c), 109 µg/l (d), 25 µg/l (e); binary mixtures with E2 influenced sex ratio (more females)	[192]	
Homarus americanus	Halifax harbour, Nova Scotia,Canada,	Previtellogenic oocytes; altered steroid profiles in hemolymph and testes	[98]	Field study
Homarus americanus (larva)	Heptachlor	Reduced molting rates, altered ecdysteroid levels, elevated cytochrome P450 and stress protein levels, 0.5 mg oral (animal weight 30 g)	[193]	
Neocaridina denticulata	E2 (a), lindane (b), chlordane (c)	Lowered testosterone levels in hemolymph, 10 and 1000 µg/l (a), 0.1 and 1 µg/l (b), 0.001 and 0.01 µg/l (c)	[194]	
Oziotelphusa senex senex (adult)	Lindane	Molt inhibition, 1 µg/l	[133]	
Palaemonetes pugio	Fipronil, chlorpyrifos	No effect on endocrine-related endpoints in females (fipronil); increased ecdysteroid levels in females, 100 and 200 ng/l chlorpyrifos	[195]	
Palaemonetes pugio (larva)	Methoprene	Inhibition of metamorphosis, 0.1 and 1 mg/l	[91]	

Table 4 continued

Penaeus monodon	Lindane, phosphamidon	Retarded limb regeneration, 0.5-10 µg/l lindane, 0.1-2 mg/l phosphamidon	[196]
Penaeus monodon	Lindane, phosphamidon	Molt inhibition	[131]
Rhithropanopeus harrisii (larva)	Fenoxycarb	Inhibition of development (extended duration of larval stages), ≥48 µg/l	[119]
Rhitropanopeus harrisii	Fenoxycarb	Growth inhibition, 100 mg/l; reduction of total lipid content, > 50 µg/l	[120]
Rhitropanopeus harrisii (larva)	RH 5849	Accelerated molt, 0.1 and 1 mg/l	[90]
Rhitropanopeus harrisii (larva-adult)	(S)-Methoprene	Inhibition of larval development, 100 µg/l	[197]
Uca pugilator	Cadmium	Inhibition of ovarian growth, 1 mg /l ($CdCl_2$)	[198]
Uca pugilator (adult)	PCB (Aroclor 1242), OCDF	Molt inhibition, 8 µg/l PCB; 1.6 ng/l OCDF	[130]
Uca pugilator (intermolt)	Aroclor 1242 (a), DES (b), Endosulfan (c), 4-OP (d)	Decreased chitiobase activity in hepatopancreas and/or epidermis, 2 and 8 mg/l (a), 5 mg/l (b), 0.05 and 20 mg/l (c), 10 µg/l (d)	[140]
Uca pugilator (juvenile)	Diflubenzuron	Reduced molting frequency and altered behaviour, 20 and 200 µg/l	[128]
Uca pugilator; Uca pugnax (adult)	DDT	Accelerated limb regeneration and molting, 10 and 25 µg/l	[132]
Uca puligator (intermolt)	DEHP (a), 4-*tert*-OP (b), PCB29 (c)	Inhibition of chitiobase activity in the epidermis and hepatopancreas,50 mg/l (a), 10 mg/l (b) 0.5 and 2 mg/l (c)	[72]

Table 4 continued

Mysids			
Mysidopsis bahia (life-cycle)	Methoprene	Reduced reproduction, ≥2 µg/l; reduced growth (weight), 62 µg/l	[124]
Neomysis integer (juvenile)	TBT	Induction of reductase activity and metabolic androgenisation, 10 – 100 ng/l; decrease in sulfate conjugation of testosterone, 1000 ng/l	[68]
Neomysis integer (juvenile)	TBT chloride	Changes in cellular energy allocation (reserves/consumption), 10-1000 ng/l	[199]

Table 5 Unpublished results of 48 h acute toxicity tests, larval development tests with *A. tonsa* expressed in mg/l (µg/l in case of DDE) and calculated acute-to-chronic toxicity ratios (ACR). 95% confidence intervals were given if possible

Test compound	**48 h acute test with *A. tonsa***		**Larval development test with *A. tonsa***		**ACR**
	LC_{10}	**LC_{50}**	**EC_{10}**	**EC_{50}.**	**LC_{50}/EC_{50} [a)]**
Ponasterone A	NOEC = 1.25		LOEC = 0.00064 [b)]		780 [a)]
Diethylstilbestrol	0.17 (0.10-0.26)	0.34 (0.25-0.39)	0.012 (0.005-0.03)	0.030 (0.02-0.046)	11
ICI 182, 780	NOEC = 2.0		1.65 (0.34-79)	1.98 (1.68-234)	≥1
Cyproterone acetate	0.008	1.5 (0.94-3.93)	0.08 (0.005-1.23)	0.16 (0.04-0.61)	10
Vinclozolin	2.5 (1.5-3.3)	5.3 (4.6-6.5)	0.64 (0.17-2.33)	3.53 1.27-9.84	1.5
p,p'-DDE [c)]	35 [c)]	83 [c)]	0.083 [c)] (0.008-0.84) [c)]	1.77 [c)] (0.4-8.0) [c)]	47
Methoprene	0.39 (0.09-0.62)	1.4 (1.12-1.98)	0.165 (0.008-0.36)	0.226 (0.014-0.35)	6
Fenoxycarb	0.09 (0.03-0.17)	0.73 (0.48-1.27)	0.003 (0.0006-0.016)	0.027 (0.014-0.052)	27
Nonylphenol acetate [d)]	2.7 (2.1-3.2)	3.6 (3.1-4.2)	0.70 (0.32-1.53)	1.01 (0.72-1.53)	4

[a)] In case of Ponasterone A NOEC/LOEC=ACR
[b)] Stimulation (30%) of larval development compared to control
[c)] Concentrations are given in µg/l
[d)] Metabolite of nonylphenol ethoxylates

5 Conclusion and Perspective

The results of the experimental work as well as the literature survey demonstrated clearly that marine copepods such as *Acartia tonsa* and *Nitocra spinipes* are suitable and very sensitive test organisms to study sublethal effects of specific toxic chemicals including compounds suspected to be potential EDCs. Their short generation times of two to three weeks allow performing partial as well as full life-cycle tests.

In preliminary studies with *A. tonsa* various parameters related to processes regulated by hormones such as growth, molting, sexual maturation, and offspring production were investigated. Endpoints were for example larval development ratio, egg production and sex ratio. Exposure experiments were conducted with naturally occurring and synthetic vertebrate and invertebrate hormones as well as compounds known to act as endocrine disrupters in vertebrates *in vitro* or *in vivo*. Larval development ratio was identified to be a remarkably sensitive endpoint. The larval development test with *A. tonsa* is rapid, cost-effective, easily to perform and responds very sensitive to low concentration exposure with a number of chemicals with the results that full concentration-response relationships are obtained allowing the determination of effective concentrations (EC_x). These altogether are big advantages compared to other frequently used (sub)chronic toxicity tests with crustaceans.

After having demonstrated that larval development ratio in *A. tonsa* was a very sensitive endpoint for evaluating effects of chemicals that might interfere with the endocrine system of crustaceans, the test has been applied to two groups of emerging environmental contaminants, brominated flame retardants and synthetic musks, known or suspected to be potential endocrine disrupters.

In doing so, important information on the chronic toxicity of these sparsely investigated groups of substances were attained, which might be of great importance for environmental risk assessment of those compounds, since the measured EC_x values were at least partly lower than any previously reported effective concentrations determined in crustaceans. For example, for the polycyclic musks HHCB and AHTN EC_{50} values of 60 and 26 µg/l, respectively, were obtained in the larval development test, which were obviously one order of magnitude lower than the corresponding 21 d EC_{50} for *D. magna*. Even the reported 21-d NOECs for HHCB and AHTN of 110 and 200 µg/l, respectively, were much higher than the EC_{50} values seen in the *A. tonsa* test.

In parallel, synthetic musks as well as brominated flame retardants were investigated in a full life-cycle test with *N. spinipes*. This test allows studying larval development ratio

as well as other individual life-table endpoints, which enable the calculation of population-level endpoints, e.g. the intrinsic rate of natural increase. In *N. spinipes*, larval development ratio has been proven to be the most sensitive endpoint too.

Though the majority of crustacean species rely entirely on sexual reproduction for their recruitment, the most often applied international guideline to test for effects on crustacean reproduction to date is based on parthonogenesis in *Daphnia magna*. Standardised methods for testing chronic toxicity with sexually reproducing species are urgently needed, particularly for the evaluation of chemicals suspected to disrupt the endocrine system. Thus, the OECD Draft Guidelines for Testing of Chemicals - Proposal for a New Guideline, Calanoid Copepod Development and Reproduction Test with *Acartia tonsa*, is a major outcome of this study, which is of particular importance for environmental risk assessment of chemicals.

Effects observed in *in vivo* tests on its own does not necessarily allow conclusions with regard to the mode of toxic action. For this reason, complementary tests with the ecdysteroid-sensitive *Drosophila melanogaster* B_{II} cell line bioassay were conducted to discriminate between general and ecdysteroid-mediated toxicity. The pentabrominated diphenyl ethers BDE-99 and BDE-100 have shown to be weak ecdysteroid antagonists. This demonstrates the capability of these PBDEs to interfere with a fundamental physiological mechanism in arthropods, a property only known for a very few substances and provides evidence that the two penta-BDEs, BDE-99 and –100 may be considered as potential endocrine disrupters in invertebrates.

When testing for endocrine disruption, it is important to remember that many effects on endocrine regulated processes can occur via mechanisms other than endocrine disruption. For example, a substantial part of crustaceans' physiology is directed towards molting, therefore, almost any chemical, which adversely affects crustaceans can directly or indirectly disrupt molting. In sublethal toxicity tests with small crustaceans like copepods, the observed effects on development, growth and reproduction are not necessarily caused by disrupting the hormonal control of these processes, but may be due to other specific mechanisms or just due to general toxicity. Hence, a chemical cannot thus be classified as endocrine disrupter, unless first certain that the chemical in question is not simply a general toxicant. To understand the toxic mode of action behind effects of potential EDCs observed *in vivo*, *in vitro* methods, e.g. receptor-binding assays, are useful. On the other hand, such assays do not taking into account processes like bioaccumulation or metabolism of xenobiotics within an exposed animal.

Therefore, there is an urgent need to develop test strategies enabling to discriminate between endocrine-mediated and other toxic effects. Further research on the endocrine system of crustaceans is needed to better understand the mechanisms inducing sublethal toxicity. The combination of *in vitro* methods and (sub)chronic copepod bioassays, as applied in this study, can provide useful information in this context.

Irrespective of the mode of action of a chemical it is simply important to know for the purpose of environmental risk assessment that there are chemicals that produce chronic effects at very low concentration levels, which will not be indicated by the ecotoxicological standard protocols typically applied. Thus, new methodologies have to be developed to overcome this fatal problem. The results of this study provide an important contribution to the transfer of knowledge from research laboratory to routine laboratory by providing draft protocols for OECD guidelines on a development and reproduction test with *A. tonsa* and a full life-cycle test with *N. spinipes*.

Finally the following approach is suggested how to proceed when testing a chemical suspected to be specifically toxic, and/or in particular to interfere with the endocrine system: The chemical to be tested is subjected to acute and subchronic screening tests (e.g. 48h acute toxicity test/larval development test with *A. tonsa*) complemented by an *in vitro* bioassay with the ecdysteroid-sensitive *D. melanogaster* B_{II} cell line. If the acute-to-chronic toxicity ratio is above ten and/or the B_{II} bioassay indicates ecdysteroid (ant)agonistic activity, a full life-cycle test e.g. with *A. tonsa,* has to be applied.

A real challenge in future research on endocrine disruption in crustaceans and other invertebrates is to get a deeper insight in their complex endocrine systems. In a recent review of nearly 75 years of crustacean endocrinological studies [51], Fingerman concluded that the work in this field “has really just begun”.

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